A DISSERTATION ON

SCREENING OF AGRICULTURAL WASTE FOR MASS PRODUCTION OF EDIBLE OYSTER MUSHROOM (*PLEUROTUS*)

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KAVITA GUPTA
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INTEGRAL UNIVERSITY, LUCKNOW
UNDER THE SUPERVISION OF
DR.RAJESH KUMAR VERMA

PRINCIPAL SCIENTIST, SOIL SCIENCES & AGRONOMY DIVISION

CSIR-CIMAP

CSIR-CENTRAL INSTITUTE OF MEDICINAL AND AROMATIC PLANTS, LUCKNOW (UTTAR PRADESH)-226015

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DECLARATION

I declare that "**Screening of Agricultural Waste For Mass Production Of Edible Oyster Mushroom (*Pleurotus*)** " is my work that it has not been submitted for any degree or examination in any other university and that all the source I have used or quoted have been indicated and acknowledgement by complete references.

The work was done under the supervision of Dr. Rajesh Kumar Verma, Principal Scientist, Division of Crop Production and Protection, CSIR-CIMAP Lucknow.

Submitted by
Kavita Gupta
Integral University,
Lucknow.

Supervisor
Dr. Rajesh Kumar Verma
Division of Crop Production And
Protection

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List of abbreviations

SNo.	Abbreviation	Explanation
1	AA	Actinomycetes Agar
2	*A.bisporous*	*Agaricus bisporous*
3	*Agaricus spp.*	*Agaricus species*
4	BOD	Bio oxygen demand
5	Ca	Calcium
6	$CaCo_3$	Calcium Carbonate
7	$CaSO_4$	Calcium sulphate
8	cm	Centimeter
9	*C.dendroides*	*Cladobotryum dendroides*
10	CFU	Colony forming unit
11	*C.multiseptatum*	*Cladobotryum multiseptatum*
12	*C.mycophilum*	*Cladobotryum mycophilum*
13	*C.varium*	*Cladobotryum varium*
14	*C.verticillatum*	*Cladobotryum verticillatum*
15	*E.americana*	*Ewingella americana*
16	Fe	Iron
17	HCl	Hydrochloric acid
18	$HgCl_2$	Mercuric chloride
19	K	Potassium
20	*L.edodes*	*Lecanicillium edodes*
21	*L.fungicola*	*Lecanicillium fungicola*
22	Mixed (P+L)	Mixed (pamarosa)
23	Mn	Manganese
24	N	Nitrogen
25	NA	Nutrient Agar
26	P	Phosphorus
27	*P.agarici*	*Pleurotus agarici*
28	*Pantoea spp.*	*Pantoea species*
29	*P.antrinopileatus*	*Pleurotus antrinopileatus*
30	*P.chrysosporium*	*Pleurotus chrysosporium*
31	*P.cintrinopileatus*	*Pleurotus cintrinopileatus*
32	*P.cystidious*	*Pleurotus cystidious*
33	*P.eryngii*	*Pleurotus eryngii*
34	*P.flabellatus*	*Pleurotus flabellatus*
35	*P.florida*	*Pleurotus lorida*
36	*Pleurotus spp.*	*Pleurotus species*
36	*P.ostreatus*	*Pleurotus ostreatus*
37	*P.pulmonaricus*	*Pleurotus pulmonaricus*
38	*P.reactans*	*Pleurotus reactans*

39	*P.saju-caju*	*Pleurotus saju-caju*
40	*Pseudomonas spp.*	*Pseudomonas species*
41	*P.talaasii*	*Pleurotus talaasii*
42	RBCA	Rose Bengal Choramphenicol Agar
43	SMS	Spent mushroom substrate
44	*Tricoderma spp*.	*Trichoderma* species
	UV	Ultra violet
45	µl	Microlitre
46	*V.volvcea*	*Volvariella volvacea*
47	Zn	Zinc

Introduction

The use of mushroom was started even before man understood the nature of other organisms (Quimio, 2014). Cultivation of mushroom was started in the ancient times for their nutritional value and flavour and rich in taste (Chakraborty 2011). Mushrooms are also known as 'white vegetables' or' a boneless vegetarian meat' which contain sufficient amounts of proteins, vitamins and fiber and certain medicinal properties (Thakur *et al*., 2013, Meng *et al*., 2016).

Mushrooms are macro fungus with a distinctive fruiting bodies which can be found growing on ground and underground .The macro fungi have fruiting bodies are large enough to be seen with naked eyes and can be picked easily with the hand (Mushworld, 2004). It requires an organic substrate as a medium which should rich in nutrients, especially nitrogen, phosphorous and potassium. The material should also to be rich in lignin and cellulose, which give nutrition to mushroom mycelium (Kimenju et al., 2009). Initially the mushrooms are classified as vegetables for many years.

Mushrooms have high content of proteins, vitamins, minerals, fibers, trace elements and low calories and cholesterol (Wani *et al.,* 2010, Rajeshbabu *et al*., 2012). Oyster mushroom was collected as a wild specimen from the Florida forest and later spread in many countries around the world as the most cultivated oyster mushroom species. Oyster mushroom is commonly grown in India under the temperature between 20-28ºC, but stops above 28^0C (Thakur *et al*., 2001). It is characterized as fast mycelial growth and high saprophytic colonization activity on cellulosic substrates. They have the ability to break-down the layer of cellulose and lignin bearing materials without any fermentation (Thakur *et al*., 2014). Mushroom cultivation implies principles of environmental engineering, microbiology and solid state of fermentation in a conversion of domestic, industrial and forestry wastes into the food for human being (Thakur *et al*., 2012). White oyster mushroom *Pleurotus florida*, is white in colour which forms primordial head formation to maturity, and this mushroom also grows in bunches. The pileus of this mushroom is with thin margins, smooth and pileus thickness is lesser as compared to *Postreatus* and *P. sajor-caju.* After *Agaricus Bisporus, Pleurotus ostreatus* is most cultivated edible mushroom all over worldwide (Sanchez, 2010). Essential materials used for the mushroom production was tree stumps, wood logs and wooden dust. In 1995 after the successful cultivation of *Agaricus bisporus* which paved the cultivation of *P.ostreatus* on wood. Today the cultivation of *A. bisporus* is widely distributed all over the world.

Bertil and Gunilla (2000) initiated Rivendell Mushroom Project at Rivendell Gardens in Shinyalu Division of Kakamega Sub-County (Formerly Kakamega District). The aim was to assist the poor farmers to create some extra income, extra food and create employment. Unfortunately, the project was collapsed immediately the initiators left their

country to Sweden. Then the idea was later adopted at Vihiga sub-County formerly Vihiga district by a community based organization known as Vihiga Mushroom Project (Vimpro).The production of mushroom was completely different from those of plants and it is a big group of fungi. Mushrooms are saprophytic in nature because they have no chlorophyll for the synthesis of carbohydrates and food. People are collecting them from the wild for ages and cultivating them as a valuable food. These mushrooms have cap, gills, stalk or stipe, spores, ring, volva and mycelium (Thongnaitham, 2012).

There are about 10 to 14 thousand species of mushroom that cover all types of mushrooms worldwide including edible and medicinal mushroom. Among them about 2000 of these mushrooms are identified and are edible, and about 20-60 species of mushrooms are commercially cultivated (Maria Florence and Balasundaran, 2000; Cheung, 2008; Marshall and Nair, 2009; Dündar, Yildiz, 2009; Patel et al., 2012) and many of these species are collected from the wild. People who are aware of the nutrition values of mushroom have started its growth for supplement, the amount harvested from the forests (Marshall and Nair, 2009). Now, mushroom production is very common all over the world. Twelve species are grown for food and medicinal purpose; some species being:

Agaricus bisporus, Lentinula edodes, Pleurotus spp., Auricularia spp., Volvariella volvacea, Flammulina velutipes, Tremella fuciformis, Hypsizygus marmoreus, Pholiota nameko, Grifola frondosa and *Coprinus comatus.*

Most of the researchers report that mushroom contains many beneficial substances, such as carbohydrates, proteins, fats, minerals, and various vitamins like vitamin B complex. Scientific research works showed that mushrooms are considered equal to that of meat in nutritional values. Commonly cultivated species in the world is *Agaricus bisporous* and *Lentinula edoles* and the third common species is the Oyster Mushroom. The oyster mushroom is an important and commercially cultivated for food and its production is increasing day by day all over the world.

Scientific classification of oyster mushroom according to Randive (2012) is as the following:

Kingdom – Fungi
Phylum – Basidiomycota
Class - Agaricomycetes
Order - Agaricales
Family - Pleurotaceae
Genus – Pleurotus

There are many species of *Pleurotus* identified all over the world. Most of them are suitable for cultivation. Some *Pleurotus* species are *Pleurotus ostreatus*, *Pleurotus columbinus, Pleurotus florida, Pleurotus salignus, Pleurotus spodoleucus*, *Pleurotus pulmonarius; and* subspecies are *Pleurotus sajor-caju, Pleurotus sapidus*, *Pleurotus*

populinus, Pleurotus cornucopiae, *Pleurotus djamor*, *Pleurotus flabellatus*, *Pleurotus eryngii*, *Pleurotus* cystidiosus, *Pleurotus* calyptratus, *Pleurotus dryinus*, *Pleurotus purpureo-olivaceus* and *Pleurotus tuber-regiu*. However, among them the most important cultivated species is *Pleurotus ostreatus*, because they are easier to cultivate, favorable to taste, and grow economically on different kinds of organic waste raw material (Kong, 2004).

Pleurotus spp. has a great ability to grow on variety of crop residues (Mamiro, et al., 2011) and since they are saprophytic in nature, they can easily decompose organic matters like wood, straw, plant leaves and the weed stalks found. The oyster mushrooms consumed raw material must contain carbon which include cellulose, hemicelluloses and lignin. Most of organic matters such as wheat straw, barley straw, rice stalk etc. also contain cellulose, hemicelluloses and lignin which can be used as a mushroom substrate. These mushroom substrates must be adjusted to a pH range of 6 to 8 by adding compound such as gypsum (Kong, 2004).

The life cycle of *Pleurotus ostreatus* starts as spores when they are dispersed from the gills. A single(spores) basidiospore germinate to form the mass of homokaryotic mycelium if the weather conditions are in favor of its germination and growth. This form is the same in the most of the mushrooms (OECD, 2005) and spreads in all direction within the growing medium (Atkins, 1972). After that, haploid hyphae find a mating pair and fuse together and undergo plasmogamy, and then dikaryotic mycelium can be seen. Only the diploid hyphae produce fruiting body under suitable temperature and moisture conditions. In such case, mushroom starts the vegetative part (Shimelis, 2011). In the ventral cap of the mushroom next basidia are produced on the gill; two different nuclei fuses together and result in karyogamy. At last the diploid nucleus goes through meiosis, and the haploid spore in the basidia can be dispelled through the wind (OECD, 2005). Then, the life cycle repeats (figure 1).

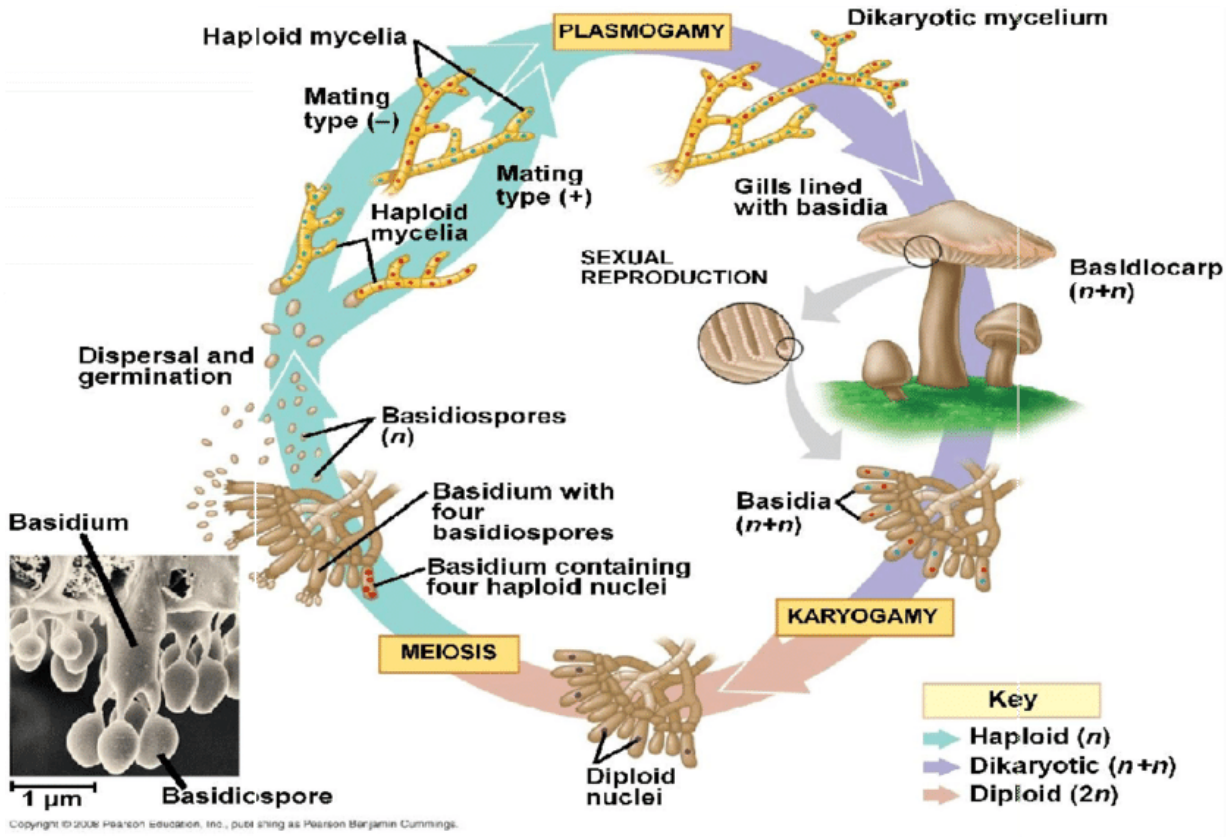

Figure 1-Life cycle of the oyster mushroom

The requirement of oyster mushroom varies at the various stages of its growing period. The optimum temperatures required for the mycelia growth and formation of pinhead are between 20 to 30^0C and 10 to 20^0C, respectively. The moisture of substrate should be maintained between 60-70 % and during the time of fruiting moisture should be between 80-95% because 80% or over of the fruit body is water. *Pleurotus ostreatus* required low level of light during its cultivation and it is different from the other cultivated mushrooms. Oyster Mushroom grew naturally in the forest and since the light of forest is bluish, fluorescent light can be used for lighting purpose (Kong, 2004).

Many methods and techniques are used for preparation of substrate to make composting like oyster mushroom directly grow on the substrate thus composting may not be necessary (Atkins, 1972)but some people compost the substrate in mushroom growing farms, because they cultivate two different mushroom species such as *Agaricus spp*, and oyster mushroom. Oyster mushroom (*Pleurotus ostreatus*) is a magical crop which are to be cultivated in the developing countries. Firstly, Oyster mushroom can be grown easily on variety of agricultural residues. Secondly, they are useful for cleaning and recycling the environment. Oyster Mushroom was collected from the nature along with several kinds of other mushrooms species by the people of

Turkey, North Cyprus and North Iraq. Generally people prefer this species much more than the other species of mushrooms. People are demanding *Pleurotus ostreatus* to make food in North Cyprus and North Iraq. Due to this reason it was more expensive than the other mushrooms. These mushrooms, are cultivated outdoors on the logs and indoor on different waste raw materials which includes wheat, barley, corn, cob and others in the Middle East. The indoor cultivation of *Pleurotus ostreatus* has gradually increased. The conditions which are required for the growth of mushroom have to be observed and managed well. Some critical conditions adversely affect mushroom production includes temperature and relative humidity in all phases of mushroom growth such as spawn running, fruit induction and harvesting.

The *Pleurotus ostreatus* consists of white, cream, yellow, pink or brownish color with thick fleshy caps having eccentric or lateral stipes. They grow on logs or lignocellulosic residues of agricultural and forestry wastes. Oyster Mushroom are related to some of the species of mushroom such as *Pleurotus ostreatus*, *Pleurotus pulmonaris*, and *Pleurotus populinus* are similar in many ways, but in the DNA analysis they have few differences, if the differences is morphology and ecology that can be observed in the field are not clear (OECD, 2005) (see figure 2).

Figure 2-Morphology of *Pleurotus ostreatus*

It has been reported that the spent mushroom substrate contain nutrients, useful for plants growth. These materials are nontoxic for the cultivation of plant crops and can be used as soil amendment for crops systems (Jonathan *et al.,* 2014). In the crop system of growing plants, SMS was degraded further in the soil humus, important to maintain soil, water holding capacity, maximizing the productivity of food crop, good aeration.

The addition of spent mushroom compost to agricultural field, it has been found to be an effective soil manure and conditioner and also have been found to increase the yield of some leafy vegetables crops. The spent mushroom substrate has potential to bio-remediate for several agricultural grade fungicides and pesticides (Ahlawat *et al.,* 2011). Spent mushroom substrate (SMS) is also used to reduce and suppress the disease of plants. Many plant diseases caused by fungal pathogen like *Pythium*-damping off, apple scab, cucumber anthracnose have been found to be suppressed by using water extract of SMS treatment (Parada *et al*., 2012). Kwak *et al*., (2015) used water for the extractraction of SMS as an eco-friendly disease control agents. Different agro-wastes like rice straw, wheat straw, sugarcane bagasse, lemon grass, rice husk and variety of other waste have been used to produce oyster mushroom (*Pleurotus spp*) and to compare mass and production of Oyster Mushroom.

Rice husk waste was chosen as the growing substrate for oyster mushroom cultivation because it contains cellulose, hemicelluloses, and lignin which are required by the fungus. Sago pulp and rice husk are the waste which are widely available, which can also use as a growing medium. The composition of sago pulp and rice husk was the best growing media for the growth and development of white oyster mushrooms. So there are a new alternative growth media that can be used for the cultivation of mushrooms and can increase the types of raw materials as an oyster mushroom growing medium.

The plants such as Eucalyptus, Neem cake, Citrus lemon, Lemon grass medicinal plants contain many substances as antibiotics, antiseptics, phenolic compounds, alkaloids (Bhonde *et al,* 1999), tannins, volatile oils, triterpenoids (Johnson, *et al,* 1996), antibacterial, antifungal (Steinhauer, 1996) and antiviral compounds. Selection of medicinal plants increases the yield of Oyster mushroom by inhibiting the growth of pathogenic microbes. The cultivation of oyster mushrooms using different substrates (sawdust, wheat straw and leaves). The mixing ratios were: sawdust and wheat straw (50%:50%), sawdust alone (100%), sawdust and leaves (75%:25%), wheat straw alone (100%),wheat straw and leaves (50%:50%) and finally leaves alone (100%).The maximum yield of 648.5g was obtained from sawdust, therefore it was suggested for the production of oyster mushroom production.

The rice straw and wheat straw have high porosity and also that it dries up very fast and easily. 20% (w/w) of cotton seed hull, added to rice straw substrate resulting in a mushroom yielding of about 20% and Wang (2010) reported that about 28–56% (w/w) of cotton seed hull was added to substrate of wheat, this result increase in mushroom yielding 15–18%, increase in yielding can be occur due to several factors .High level of nutrients provide more energy for mycelia growth and primordial formation. Supplement of cotton seed hull increased water holding capacity and can reduced mortality of young fruit-body due to water shortage.

AIMS AND OBJECTIVES

The main objectives of current study are –

a. Screening of agricultural waste as a substrate for edible mushroom.
b. Evaluation of oyster mushroom for mass production and their growth potential.
c. Analysis of quality parameters of selected substrate.

Literature Review

Mushrooms are a widely distributed food resource on earth and have been consumed because of their nutritional value and medicinal properties for over 2000 years. For their enjoyable flavor and taste, human health was improved by mushrooms due to their nutrients, including digestible proteins, carbohydrates, fiber, vitamins, minerals, and antioxidants (Acharya *et al.,* 2017, Zhang *et al*., 2016).

Mushrooms are becoming more important in our diet due to their nutritional value, high protein and low fat energy contents. The mushroom protein contains all the nine essential amino acids required by humans. In addition to their good protein content, mushrooms are a relatively good source of the other nutrients like phosphorus, iron and vitamins, including thiamine, riboflavin, ascorbic acid, ergosterol, and niacin. Mushrooms are the sources of bioactive substances such as secondary metabolites (organic acids, terpenoids, polyphenols, sesquiterpenes, alkaloids, lactones, sterols, metal chelating agents, nucleotide analogs and vitamins) glycoproteins and polysaccharides, mainly 1, 2-glucans. Due to the presence of biologically active compounds of medicinal value they are used as anticancer, antiviral, hepatoprotective, immune potentiating and hypocholesterolemic agents.

2.1 World scenario of Edible mushroom production

Among the numerous species of mushroom, Oyster Mushrooms are more advantages over other mushroom in terms of easy for cultivation, role in biodegradation and bioremediation, extracellular enzymes production and neutraceuticals production (Rashad *et al*., 2009). *Pleurotus* species, commonly known as oyster mushrooms, are edible fungi cultivated worldwide especially in south East Asia, India, Europe and Africa (Mandeel *et al.,* 2005). Oyster Mushroom is the third largest commercially produced mushroom in the world. *P. ostreatus* is the second largest cultivated mushroom species next to *A. bisporus* in the world market. Although edible mushrooms represent an important agricultural product worldwide, only a few of them (*Agaricus*, *Lentinula, Pleurotus*, *Auricularia*, *Volvariella*, *Flammulina, Tremella* and a few others) can be cultivated.

2.2 Histological Information

Like other type of edible mushroom, oyster mushrooms have been collected in the wild for many centuries. Cultivation of these mushrooms only began in the early 1990's. Early techniques and methods for growing *Pleurotus* involved tree stumps and logs as substrate, mimicking their growth in nature (Lvors 2003). In Germany in the 1950's successful attempts to grow oyster mushroom in sawdust became a historic milestone for mushroom cultivation. Mass production of oyster mushrooms first started in the last

1960's using a sawdust based substrate (Chang and Hayes 1978). Popularity and production of the oyster mushroom has been increasing ever since.

Wheat straw is a common substrate for oyster mushroom cultivation in the continental US, while the abundance of rice straw available in China is utilized as substrate (Chang and Hayes 1978). Other substrate used successfully include cotton waste, corn cobs, palm fronds tea, tea waste and peanuts shells (Cohen et al.2002, Thomas et al.1998, Kalita and Mazumder 2001, Philippouusis et al. 2001).

2.3 Taxonomy and Morphology

Species of *Pleurotus* are wood-inhabiting ligninolytic white-rot Basidiomycetes belonging to the other Agaricales. There are over 30 species of *Pleurotu*s Mushroom. Although *Pleurotus* are considered saprophytic fungi, they have been known to grow parasitically on trees as well. Fruit bodies range in color from blue-gray to white, to gray-brown and are mostly shell or spatula shaped, with a noncentral stalk. Gills are thin, broad, dense, continuous to the upper part of the stipe (stalk), and vary in color from white to light grey. Spores of *Pleurotus spp*. Range in colour from white to buff to grey-lilac, and are often produced in large quantities, sometimes provoking allergic or irritation reaction in growers.

2.4 Nutritional and Medicinal Value

Mushrooms have extremely high moisture content. Fresh mushrooms contain approximately 90% water; when dried, they contain from 5 to 20 %. However, conditions during growth, harvest, and post-harvest storage affect moisture content (Crisan and Sands 1978). Oyster mushrooms provide good nutritional value. (Yang et al .2001) reported that crude protein content on dry weight basis, as 15.4% and 23.4% in *P.cystidious* and *P. ostreatus* respectively. They contain about 60% carbohydrates (dry weight); within ranges for other edible mushrooms (Crisan and Sands 1978, Bano and Rajarathnam 1988). In addition, they were reported to be low in fat (2 to 3% by dry weight), a good source of essential amino acids and contain approximately 5 to 9 % of fibre (Yang et at. 2001).

Literature does provide good references for oyster mushroom nutritional values. However it has been demonstrated that substrate contributes to variation in nutrient value of fruit bodies (Crisan and Sands 1978). Specifically, Patrabansh and Mandan (1997) found that the mineral content, specifically P, Mg, Fe, Mn and Zn of *P.pulmonarius* fruit bodies increased when grown on substrate with higher mineral content. Substrate composition has also been shown to influence fruit body flavor. Oyster mushroom (*P.flabellatus*) grown on rice straw fruit body flavor. Oyster mushroom (*P.flabellatus*) grown on rice straw supplemented with cotton seed were reported to

have a distinctly different flavor compontent concentration than those grown on unsupplemented straw (Bano and Rajarathnam 1998).

2.5 Physiology

Like other white rot fungi, species of *Pleurotus* secrete an arsenal of enzymes specific for the digestion of lignocelluose materials. Degradation of substrate, including types and quantities of enzymes produced, differs among different species of white–rot fungi and different growth conditions (Free and Destroy 1982, Boyle et al. 1992). Busell et al. (1996) has studied and summarized the enzymatic profiles of three edible mushrooms, *L.edodes* grows naturally and is cultivated, on high lignin substrates such as wood logs and sawdust. This fungus is known to produce both Mn peroxidase and laccase, two enzymes specific for lignin degradation. The paddy straw mushroom prefers high cellulose substrates such as straw. It produces many celluloytic enzymes but none of the lignin-degrading enzymes production was quantified, *P.pulmornarius* produced higher levels of both cellulotyic and ligninolytic enzymes (Buswell et al. 1996). Specific lignin-degrading enzymes produced by *Pleurotus spp.* include lignin perxidase, Mn peroxidase, and laccase (Orth et al. 1993). Kaal et al .1995). Celluloytic enzymes of *Pleurotus spp*. include endoglucanase, exoglucanase, ß-gluccosidase (Busell et al.1996, Tan and Wahab 1997) and cellobihydrolase (Tan and Wahab 1997 , Velazuez-Cedeno et al .2002).

Inorganic nutrient effect, particularly Ca, Mg, Mn, Fe, and Zn, on lignin degradation by *P.chrysosporium*, was studied by Jeffries et al. (1981). It was found that Mn levels had a strong influence on lignin degradation, rates being greatest when Mn was removed. Increased levels of Mn resulted lignin degradation inhibition, however, inhibition was alleviated by increased concentration of either Ca or Mg, Iron and Zinc did not shown a major effect or lignin degradation. Lignin, cellulose and mineral contents of substrates have also been shown to influence growth and fruiting of *Pleurotus*. Philippoussis et al. (2001) demonstrated that the cellulose lignin ratio of the substrates was positively corrected to the mycelia growth rate and mushroom yield of both *P.ostreatus* and *P.pulmonarius*. Fasidi and Olorunmaiye (1994) verified that certain macroelements and trace elements are essential for *Pleurotus* growth. Lipid cultures of P, tuber-regium showed significant decreases in myceial growth when potassium (K) was removed from the media, also and when Ca was removed from the media and also when Ca was removed. Additionally, medium supplemented with Cu, Fe, Mn and Zn produced greater fungal growth than basal medium.

2.6 Production Overview

A simplified life cycle of the oyster mushroom can be separated into two biological stages: the vegetative phase, consisting of mycelial expansion and maturation, and the

reproductive phase of fruit-body production. An initial mycelia culture can be obtained from a pre-existing stock culture or through tissue mycelia culture. These methods yield more predictably performing cultures than those grown from spores (Royce 2003). Cultivation begins with propagation of mycelium on sterilized cereal, creating spawn. The spawn is used to inoculate the mushroom substrate. Once the sterilized substrate has been inoculated, it is allowed to incubate. During the incubation also called spawn run, the mycelium grows throughout the substrate and matures. If environmental conditions are adequate, the mature fungus will progress to the reproductive phase. Primordia will form and develop into harvestable mushroom.

2.7 Fruit-body Initiation and Production

Like the cultivation of *Pleurotus spp.* and all white-rot fungi, several factors are critical for successful fruiting. Certain environmental conditions are required to cue the organisms into the reproductive phase. Moisture, temperature, gas exchange and light are involved in mushroom development. When choosing or creating a site for mushroom production, consideration of environmental conditions is important.

2.7.1 Moisture

Extremely high humidity (90-100%) is recommended for optimal primordial formation. Once primordia have formed, humidity should be lowered to 85 to 90 %. The humidity levels should be managed so that mushrooms are regularly receiving moisture but excess moisture can evaporate from fruit body surfaces. Excessive moisture can cause lack of oxygen in the substrates, as well as encourages certain contaminates. Inadequate moisture can prevent primordial formation and stunt fruit body growth.

2.7.2 Temperature

Oyster Mushrooms are able to grow and thrive in a wide range of temperature environments. (Staments 2000) recommends temperature between 10-21^{0}C for environment of oyster mushrooms. Pettipher (1987) achieved successful fruiting of *P.ostreatus* with daily temperatures ranging between 8 and 33^0 C.

2.7.3 Gas exchange

Since growth of the fungus produces carbon dioxide as it decomposes the substrate, introduction of ‘outside’ air reduces carbon-di-oxide build up and increases oxygen levels. Fungal mycelium is extremely tolerant of carbon-di-oxide, thriving at 20% CO2 levels. Oxgyen is required for formation of fruit bodies. A significant decrease in ambient CO2 level and increases in oxygen is critical for the initiation and development of primordia. Thus sufficient air circulation within the mushroom fruiting site is vital.

Excessive influx of outside air, however, greatly affects both temperature and humidity of the environment.

2.7.4 Light

As a forest-dwelling mushroom, indirect natural light is considered ideal for the formation of *Pleurotus spp.* fruit bodies. Although the mycelium of the oyster mushroom does not require light, proper fruit body formation requires moderate light .Too little or too much light can lead to discolored, malformed fruit bodies or the inability to fruit. Kalberer (1974) found that oyster yield was maximized using light levels of 60 to 86 µmol/m^2/ sec (300 to 430 lux) for twelve hour days. Stamets (2000) recommends levels around 200 to 300 µmol/m^2/sec (1000 to 1500 lux) for commercial production.

2.8 Factors Affecting Yield

Species of *Pleurotus* are very efficient at breaking down lignocellulotic waste. Almost 50% of the substrate by mass is liberated as CO2 gas 20% is lost as water, 20% remains as spent substrate and 10% is converted to dry mushrooms (Staments 2000). Certain techniques are used to achieve high production rates and yields.

2.9 Spawn Rate

Grain spawn provides many points of inoculation and a nutritional boost to the substrate. Spawn rate is the amount of spawn used to inoculate the substrate and is defined ratio of spawn to substrate and is defined as the weight ratio of spawn to substrate. For example, a spawn rate of 5 % would entail using 50 grams of spawn (wet weight) for every 1000 grams of substrate. Increasing the amount of spawn used in inoculation greatly increases yield and accelerates the rate of mycelial growth and colonization, which decreases spawn run time and showed that increasing spawn rate from 1.25% to 5% resulted in yield increase of approximately 50% for *Pleurotus* and decreased spawn run by more than seven days. Faster colonization is also advantageous in deterring fungal competitors, by decreasing the window of opportunity for contaminates to establish. Since spawn must be made or purchased, spawn rate will affect production costs of cultivation. Optimal spawn rates will vary depending on mushroom species, substrate types, and cultivation conditions.

2.10 Substrate Composition and Supplements

Though variation exists among different species of *Pleurotus*, composition of substrate does play a role in vegetative and fruit-body growth. Lignin, cellulose and hemicellulose availability plays a key role in the growth of wood-decaying fungi. Philippoussis et al. (2001) determined that the cellulose: lignin ratio of a substrate was positively correlated to rate of mycelial growth and mushroom yield of both *P.ostreatus* and *P.pulmanarius.*

Research on two other species of oyster mushroom (*P.cintrinopileatus* and *P.florida*) concluded that wood based substrate rich in cellulose supported higher yield and resulted in more nutritious mushrooms than substrates of other agricultural waste (Kalita and Mazumder 2001).

Enhancing base substrate with nitrogen rich supplement is common practice to increase yields in mushroom cultivation .Commonly used supplements are grain products, such as bran and meal. The lignin degradation rate of alder sawdust by *P.chrysosporium* was shown to increase from 5.2% to 29.8% with the addition of only 1.2g/kg N (dry weight) (Yang et al.1980). That of hemlock sawdust was increased 2.2 % to 3.9 % dry weight (Yang et al. 1980). Boyle (1998) showed that most N-containing supplements increased the growth of several white rot-fungi. Yield increases using supplements with *Pleurotus comucopiae*, commonly known as golden oyster mushroom. However, addition of N supplement makes the substrate more suitable for competitor fungi and bacteria. Along with additional cost to production, use of supplements creates the need for stricter sanitation (Stamets 2000). As for other nutritional components, Boyle (1998) showed that addition of simple carbohydrates, vitamins, and micronutrients (other than N) had limited effects on growth rates.

2.11 Spent mushroom Compost as Soil Conditioner and Organic Fertilizer

Spent mushroom compost is a noxious by product of mushroom farming (Tuhy, 2015 Jonathan *et al.,* (2012) considered SMS as remnant substrate of cultivate mushroom. The use of organic manure such as spent mushroom substrate in growing agricultural crops especially leafy vegetables has been recognized in recent times as a possible means of enhancing sustainable agriculture or sustainable production of food crops (Okokon *et al.,*2009). According to Akanbi *et al.,* (2015) organic fertilizer improved cell activity, enhance cell multiplication and enlargement of fluted pumpkin. Organic manure is known to be capable of activating diverse microorganisms which release hormones like substances that stimulate nutrient absorption and plant growth (Arisha *et al.,* 2003). Spent mushroom compost is rich in organic matter and constitutes an important source of macro-micro nutrients for plants and microorganisms thereby increase the soil microflora, soil biological activity and enhance soil enzyme activity (Debosz *et al.,* 2002).

Material and Method

3.1 Material Required

- ✓ Culture (PO, BT, HU, PF)
- ✓ Substrate (sugarcane, wheat straw, rice straw, turmeric, lemon grass, pamarosa, rice husk, basil husk, basil stem)
- ✓ Cereal grains
- ✓ Laminar air flow
- ✓ Autoclave

3.2 Fungal Culture

The pure culture of *Pleurotus ostreatus, Pleurotus sajor-caju, Pleurotus djamor*, *Pleurotus florida, Calocybe indica* and *Agaricus bisporus* was obtained from the Rahmankhare , Lucknow, India. The cultures were maintained on Potato Dextrose Agar (PDA) and 2% Malt Extract Agar (MEA) slants and stored at 4^0C.

3.3 Maintenance of culture

The culture of *Calocybe indica (*milky mushroom*)* was stored at 15^0C and the culture of *Agricus bisporus, Pleurotus ostreatus, Pleurotus sajor-caju, Pleurotus djamor and Pleurotus florida, P. flabellatus, P. eryngii*, was stored at 4˚C using a refrigerator. For the maintenance of pure line of culture, the subculturing was done after every 35 days of intervals**.**

3.4 Preparation of pure culture media

Besides infrastructures, raw materials and equipment's healthy vegetative mycelial culture is required to produce quality spawn .The vegetative mycelium is raised on to a convenient culture media. A variety of culture media can be used to grow vegetative mycelium before it is inoculated on a suitable substrate to grow mushrooms. The culture media are also used as substrate for inoculation, multiplication, maintenance and preservation of mushroom cultures.

3.5 Potato Dextrose Agar (PDA)

Peeled and diced potato - 200g

Dextrose - 20g

Agar – agar powder - 1 litre

pH - 7.0 pH

Potatoes are peeled, washed and cut into small pieces of about 3-4 cm and boiled in water for around 20-25 minutes. The boiled potatoes are extract and filtered through a cheese cloth and potatoes cubes discarded. Raise the volume to 1 litre. Mixed the Dextrose and agar-agar powder by stirring and pH is adjusted.

NOTE – Green part of potatoes should not be used because they contain anti-fungal alkaloids, that may harm the mushroom mycelium.

If the pH of the medium is below 6.5, N/10 NaOH is added (drop by drop) to raise it to 7.0 pH. Whereas, if the pH is above 7.0 N/10 HCl is added to bring it down .The pH should be adjusted to 7.0 before sterilization. It has been noticed that the ph declines approximately 0.5 upon autoclaving. After autoclaving the pH comes to 6.5 which can suitable for the growth of mycelium**.**

If the culture is to be raised in Petri plates then the medium is first sterilized in 500 ml conical flasks, 15-20 ml of lukewarms sterilized culture medium is poured in each pre-sterilized petri-plates. If the cultures are to be multiplied in test tubes then the medium is poured in large number of cultures tubes @ 15 ml / tube.

The cultures tubes or conical flakes should be plugged with cotton (non-absorbent) and should be autoclaved under moist steam at 15 pounds per square inch (p.s.i.) pressure for around 25-30 minutes. A pressure of 15 lbs psi eliminates all microbes , which later compete with vegetative mycelium of desired mushroom .For the proper mycelium growth of a mushroom, cultures tubes should placed in slanting position in order to provide more surface area after sterilization .

Allow the culture medium to solidify in cultures tubes or in petriplates for few hours before they are used for sub-culturing. Sterilized the petriplates in an oven at 180^0C for about 2.5 hours and allowed to cool down at the room temperature before they can use for plating media.

3.6 Isolation of fruit body

Culture medium should be sterilized both in petriplates and culture tubes. Scalpel, inoculation needle, wide mouth test tubes, spirit lamp, scissor, forceps, match-boxes, 70% ethanol(rectified spirit), laminar air flow, fresh basidiocarps (fruiting -bodies) should be there in an inoculation room. In the inoculation room there should have double door entry or air curtains at the entry points in order to cut forceful entry of air inside the room .Many a times contamination can enter through air currents entering into the inoculation room. The laminar air flow has micro-filters that eliminate all microbes and dust particles present in the air and surrounding. When the laminar flow is on, all dust particles in the air and microbial contamination, more than 4 microns in size are retained with the help of filter and clean air is blown in a working place.

3.6.1 Precautions during isolation of fruiting bodies

Before working in the laminar flow the UV lights should be on in order to eliminate microbes and contaminations. All material should exposed to UV light to eliminates contamination present on the surface of culture of culture tubes, petriplates, forceps, scissors and the elements used in laminar flow except the living cultures and fruit . Before using the laminar flow keep in mind to switch off the UV light because this light is harmful to all living organism including human beings, cause skin cancer and can even cause mutation. While entering into the inoculation room must wore the foot ware and apron and these wares should not be used outside the spawn lab.

Before starting the inoculation, or any type of work in laminar flow, the surface area should be wiped out with cotton dipped in rectified spirit. If in case the hands are come in touch with UV light, it must be washed with rectified spirit (70% ethanol).

3.7 Preparation of cultures by tissue culture or multimedia spore cultures

3.7.1 Tissue culture

In case of *Agaricus* species (button mushroom), a young fruiting body should be harvested freshly and brought to the laboratory for tissue culture or for futher experiments .The fruit body should be well cleaned and casing soil should be removed with the help of cotton swab .

The fruiting body is held with inoculation needs and dipped in 0.1% mercuric chloride ($HgCl_2$) or 70% ethanol for around 30 seconds to kill microbes present on its surface.

The scalpel surface is sterilized by dipping in 70% ethanol and then heat it on the flame. After scalpel gets cooled, the fruiting body is spilled longitudinally and bits of tissue approximately 3-5 mm are cut from pileus-stipe junction (collar region). The transferred of bits are aseptically done onto pre-sterilized petriplates containing convenient culture medium with the help of a sterilized forceps. These pertiplates are wrapped with the help of parafilm and incubated in the BOD at 25^0C for a week. The growing mycelium from the side of edges of about 5mm in size with culture medium should be aseptically transferred to a number of test tubes.

Incubate these test-tubes in BOD for 2-3 weeks. This pure culture can be used as a inoculums for mother spawn preparation. In case of *Pleurotus spp.,* the tissue culture is raised from the junction of pileus and stipe portion. Rest of the procedure is same as above. In case of tropical mushrooms like *Volvariella* and *Calocybe* species the petriplates and test tubes containing bits of fruiting body are incubated at 30-32^0C not in 25^0C.

(Control and Mother Spawn)

Figure 3-Culture slants showing culture medium and pure mycelium cultures

3.7.1.1 Spore culture

There are difference in the sexuality pattern in cultivated mushrooms; *Agaricus bitorquis*, *Lentinula edodes* , *Pleurotus spp*. and *Auricularia spp*. are heterothallic, whereas *Agaricus bisporus* and *Volyariella* are secondary of primary homothallic species . Direct single spore culture may not result in fruiting in hetero-thallic species. Therefore, in spawn labs, the pure mycelial cultures are raised from tissues cultures. Nevertheless, from any mushroom species for spawn preparation multi-spore cultures can be raised. It has benefits, for raising a spore culture, mass of spores known as spore print is required. The print spore can be obtained from a fruiting body on to a sterilized petriplate or plain paper. In case of *Agaricus*, fruiting body, a healthy whose veil is still intact is harvested and cotton swab is used to remove the casing soil. Bell jar, Petri plates and beakers are sterilized is an oven at 180^0C for 2.5 hours before a spore print taking. The fruiting-body is mounted on the coil and kept in an open petriplate and covered with the help of beaker and in a bell jar it should be enclosed for around 48 hours. Inside the petriplates or on a plain paper millions spores are shed off. These spores print can be stored at dry place until used. In case of *Pleutotus spp*, Spore print can be gain by placing the mature fruiting body with gills facing on the paper side, in a petriplates or on sterilized plain paper within 48 hours. Pure cultures from spores can be raised in the following two ways:

3.7.2 Multi spore culture

For raising pure culture multi spore culture is also used. From a mass of germinated basidiospores it is raised. Using sterilized inoculation needle, a loop full of spore mass is picked from a spore print. Suspended 10 ml sterile distilled water in 18×150mm test tube and mixed them properly. 1 ml of this suspension is remixed in 9 ml of sterile water. Again on petriplates 1 ml of diluted spore suspension is spread with the help of a glass rod containing 20 ml of a convenient culture medium. Incubate these petriplates inside the BOD incubator at 25^0C for a week. The colonies fast growing multi-spore are picked up with piece of agar-agar and transferred them to freshly prepared culture medium in test tube under aseptic conditions. Incubate these test tubes for 2-3 weeks in a BOD incubator. Now multi spore culture is ready for inoculation in the wheat grains or other cereal grains for preparation of spawn.

3.7.2.1 Single spore culture

In secondary homothallic species of mushroom such as *Agaricus bisporus* only around 65% of its spores are fertile rest of them are sterile .In such cases identification of fertile spores can be done for cultivation purpose whereas a species of *Pleurotus*, *Lentinula* , *Auricularia* and *A.bitorquis* are heterothallic and single spores are not fertile .In such cases single spores are not used for preparation of culture and spawn and they may not result into fruiting bodies .Single spores are immense in developing into new varieties , hybrids and modern breeding techniques are used by single spores selections .The process of raising a single spores cultures is as same as multispore culture. Single spores in such cases are not used for preparation of culture and spawn, as they may not eventually result into fruiting. Nevertheless, single spores are of immense importance in developing new varieties, hybrids and single spore selections using modern breeding techniques. The process of raising single spore cultures is the same as that of multispore culture. In single spore isolation, spore suspension is again diluted to get a concentration of 20-30 spores before spreading in petriplates. Only germinating single spore mycelium should be marked carefully, picked up and transferred to sterile test tubes, instead of picking up a growing multispores culture .These tubes should be incubated for 2-3 weeks in a BOD incubator. The single spore cultures colony will growing slow and can be identified visually.

3.7.3 Sub-culturing

A pure vegetative mycelia culture is established as a result of isolation and purification. For their use in inoculation of grain substrate for production of spawn in a large scale and use in further purposes these cultures are maintained. Sub-culturing can be done by transferring a small piece of growing pure culture on a suitable medium. In order to maintain vigour of the mycelium culture media should be changed in subsequent sub-

culturing. Cultures should be kept in a refrigerator at 4^0C for 2-3 months and again sub-culturing can be done for making mother spawn.

Figure 4-Pure culture of *Pleurotus ostreatus* on petriplate

3.8 Maintenance and conservation of stock cultures

There are various method of the maintenance and conservation of mushroom culture and a good culture collection centre to adopt more than one method for their preservation. Mushrooms might be of academic, industrial, medicinal or of horticulture importance .When a new genus on a species is discovered, it is generally deposited in established germplasm bank. This ensures the availability of the organism to use them in future need. In addition, mushroom strains are having industrial importance, patented are preserved and availability of such strains has become restricted (Jong and Birmingham, 1991). There is no suitable method to check and evaluate the quality of mother spawn by rapid on spot examination. If no degenerative changes taken place during the preparation or maintenance cultures and spawn of mushroom, then the preservation have been relatively simple. Unfortunately it is not true. If the culture and spawn get degenerated there is loss of desired traits leading to allow growth and development, poor rate of survival and also productivity level get lowered (Chang and Miles, 1989).

Through a sexual process spores of heterothallic or secondary homothallic species are produced which are having a genetic differences (Petersen, 1995). Spores of heterothallic or secondary homothallic species are produced through a sexual process will have genetic differences. Spores of primary homothallic species expected to have

similar genetic .Only *Volvariella* basidiospores are primarily homothallic but still shows some variations (Chnag et.al., 1981). Mating tests for homothallic species would require if single spore cultures are maintained as well as tests for ability of fruiting. It would be important to check culture fertility by fruiting testing regularly. In general the vegetative mycelia of only known origin are stored Variety of methods are available for the conservation of mushroom culture suitable for a particular need after pure culture are obtained e.g. preservation for shorter or longer period .The preservation method depends upon various factors but the availability of necessary equipment's and funds is commonly a determining factors in such decision.

3.9 Frequently sub – culturing

Most of the mushroom mycelium continues to grow under the recommended temperature and proper ph conditions until the nutrients of suitable culture medium are exhausted .These culture are viable only for few months depending upon following factors such as growth rate, substrate and method of storage and etc .After obtaining optimum growth of mycelium, cultures of mushroom are stored until sub-culturing become necessary. Cultures are prepared on slants in test tubes or in culture bottles for storage purposes .Cultures can be stored at room temperature for a few weeks. Sub-culturing period can be extended to 4-6 months by storing it at 4^0C in refrigerator. In laboratory, the sub-culturing of edible mushroom strains on suitable culture media.

In laboratory, the edible mushroom strains are sub-culturing on suitable culture media. Mushroom strain such as *Volvariella volvacea* and *Calocybe indica* are incubated at 30-32^0C for 10-15 days, respectively. The other strains of mushroom are incubated at 25^0C or 2-3 weeks until the slants get fully covered with mycelium. Once completely grown culture of *V.volvacea* have obtained, they should kept at room temperature and strain of *V.volvacea* should be sub-cultured between 6-8 weeks. Strains of mushroom such as *Lentinula*, *Pleurotus* and *Agaricus* species should be kept in a refrigerator at 4^0C and they should be sub-cultured every 3-4 months. Deviation of the culture from the original characteristics can be detected with mycelia cultures. Common degenerative symptoms are slow growth, thin mycelium, weak in appearance, fluffy or matted but has normal growth rate. If the growth of mycelium is slow it required more time for colonization to carry particles of viruses .If the mycelium is fluffy the grain are stick together properly and is harder to spread in compost as compare to normal grains .It gives lower yields and tends to form stroma . Such type of mycelia should be discarded (Chang and Miles, 1989). Cultures tubes of mushroom strain *Volvariella spp.* forms chlamydospores, are brownish in colour. If the more chlamydospores are showing on the cultures tubes, this indicates that the cultures has a good vigour and will give high yielding.

Because of degeneration and mutation during prolonged vegetative propagative of stock culture there is partially loss of mushroom forming capacity and desired quality.

Furthermore, the procedures of conservation of living fungi are time taken, a costly and risky. Ultimately the repeating of sub-culturing can result in preserving of a culture that is different from that of original one. The disadvantages of frequent sub-culturing are loss of traits which are desirable, contamination chances by air borne spores or mites carried infections, constant specialist supervision, labour intensive and time – taken process etc.

Table 1-Comparision of mushroom culture preservation methods

Method of preservation	Cost of materials	Cost of labours	Longevity	Genetic disability
Storage at room temperature	Low	High	4-6weeks	Variable
Storage in refrigerator	Medium	High	4-6 month	variable
Storage under oil	Low	Low/medium	4-5years	Moderate
Storage in water	Low	Low/medium	2-3 years	Moderate
Storage in deep freezer (-70oC)	Medium	Low/medium	4-5years	Moderate
Freeze-drying of Basidiospore	High	Initially high	20 years	Good / medium
Liquid nitrogen	High	Low	Indefinite	Good
Ultra-low mechanical freezers (-150oC)	High	Low	(-)	(-)

3.10 Mushroom repositories

To maintain and produce the reliable pure culture spawn with good qualities is a key operation and the first step in the success of mushroom cultivation. Mushroom culture repositories / banks play an important role in supply of pure and authentic culture to many of the mushroom spawn producing units .One can obtain pure cultures for making spawn from any of the National or International repositories . Some of them are listed below:

3.10.1National

a. National Research Centre for mushroom (ICAR),Chambaghat, Solan (HP) .

b. Division of Mycology and Plant, IARI, Pusa, New Delhi, 110012.

c. Indian Institute of Horticulture Research (ICAR), Bangalore, Karnataka.

d. Institute of microbial technology (IMTECH), Sector 39D, Chandigarh.

e. Department of microbiology, Punjab Agriculture University, Ludhiana, Punjab.

f. Department of plant pathology, Maha Rana Pratap Rajasthan Agriculture University Udaipur, Rajasthan.

3.10.2 International

a) American Type College (ATCC), Rockville, Maryland, USA.
b) International Mycological Institute, Kew, Sueery, UK.
c) National Regional Research Laboratory (NRRL), USDA, Preoria, Illinosis ,USA .
d) Fermentation Research Institute (Fungus culture (CCFC), Canada.
e) College of agriculture Sciences , Pennsylvania State University ,USA
f) Dutch Mushroom Experimental Station, the Netherlands.

3.11 Spawn Production

Substrate

3.11.1 Cereal grains

Mushroom spawn can be prepared by using grains like wheat, maize, bajra, jowar, rye etc .To substain the inoculum of mushroom mycelium, larger grains carry a greater reserve until it is established and feeding on the compost, so they may be more effective in poor composting or adverse conditions .On the other hand the small grains provide more points of inoculum per gram of spawn, so if all the grains of both types grow equally well, small grains will penetrates the compost sooner .

Mushroom species	**Cultivation method**	**Spawn substrate**	**Grain colonization period**
Agaricus spp.	Trays , plastic bags / shelf system	Cereal grains	20-25 days
Auricularia spp.	Wood logs / synthetic bags	Sawdust / grain	18-20 days
Lentinula spp.	Wood logs /synthetic bags	Saw dust / cereal grains /wood sticks	20-25 days
Pleurotus spp.	Synthetic bags	Cereal grains	10-15 days
Volvariella spp.	Outdoor / indoor in cages	Used tea leaves/ipil ipil leaves /paddy straw + saw dust / cereal grains / cotton waste	10-12 days

3.12.2 OTHER SUBSTRATE

Cereal grains are suitable for making mother spawn of any of the mushroom but prohibitive cost a variety of agriculture waste like corn cobs , wooden sticks , rice straw , sawdust and used tea leaves etc. have to be used . A variety of substrates for different mushroom have been suggested for making spawn, such as sawdust of red wood tree species is mostly preferred. Wheat bran ranging from 10 to 20 % is mixed with sawdust to increase the percentage of moisture. Tea leaves which are used are washed and then dried before using them for preparation of mother spawn. Ipil-ipil leaves are mixed with saw dust in the ratio 3:1 and can be used as substrate. Paddy straw can also be cut into small pieces of 2-3 cm and soaked overnight before using it as substrate for spawn.

3.13 Substrate preparation

Spawn substrate should have following desirable characteristics:

a) For the fungal colonization larger surface area should be available.
b) Cereal grains should be free from every type of disease.
c) Cereal grains should not be broken, damaged by insects or not even old.
d) Essential nutrients should be present by mushroom mycelium growth.
e) It should not any inhibitory compounds to desirable mushroom species.

The cereal grains should properly washed with water 3-4 times to remove dust , straw particles and also undesirable seeds and grasses etc .Washed grains should be soaked in water for 20-30 minutes to save energy .These grains should be taken in a wide mouth container and boiled in water for 20-30 minutes. Normally, for soaking and boiling

20 kg of wheat grains, 35 litres of water is required. Grains should absorbed 55-60% moisture after it gets boiled. Excess water should be removed with muslin cloth. Grains are allowed to leave for few hours so that water evaporated from the surface .To maintain its pH gypsum, calcium carbonate, calcium sulphate should be added .The best result are obtained by using 200g of gypsum and 50 g of calcium carbonate for 10 kg grains . Firstly calcium carbonate and calcium sulphate are mixed separately and then the mixture of both is thoroughly mixed with grains mixing should be done after wearing gloves to avoid contamination**.**

Figure 5-Boiling of wheat grains grains

Figure 6-Draining of water from boiled

Figure 7-Adding CaSO4 and CaCO3 in cereal grains for autoclave

Figure 8-Autoclaved Cereal grains are ready for the preparation of mother spawn

3.14 Master spawn preparation

Near about 300g of prepared substrate in glucose and milk bottles is filled and raised upto 2/3 volume and plugged with non-absorbent cotton .About 300g prepared substrate is filled in glucose / milk bottles upto 2/3 volume and plugged with non-absorbent cotton. Then autoclaved these bottles at 22lb psi pressure at 126 ^{0}C for about 2 hours leave those bottles in the room for 24 hours to cool to ambient temperature. A small piece of growing mycelium is transferred aseptically to these bottles and inoculated bottles are incubated at 25^{0}C. Shake these bottles on 5th and 10th days after inoculation .After that these mother spawn can be used for inoculating spawn bags after 2-3 weeks. Incoculate bottles are incubated at 22-25^{0}Cfor *Agaricus*, *Pleurotus* but at 30-32^{0}C for species of a *Volvariella* and *Calocybe*.

Figure 9-Vertical autoclave for sterilization of spawn substrate

3.15 Commercial preparation of spawn preparation

Commercial spawn can be prepared by using heat resistance polypropylene bags.

For half and one kg spawn, the size of bags should be of 35×17.5 cm and 40×20cm in size, respectively. Double sealing at the bottom of polypropylene bags should be present and after filling the grains they are plugged with the help of a non-absorbent cotton .150 gauge of polypropylene bags should be used .Sterilized these bags at 22lb p.s.i for 1.5 -2 hours in autoclave. Shake the autoclaved bags before inoculation so that

the water droplets inside the bags get reabsorbed by the grains .The sterilized bags should be kept under the UV light for about 30 minutes 10-15 grams of mother or master spawn bottles are inoculated per bags under aseptic conditions. For inoculation 25 to 30 commercial spawn bags of half kg capacity one bottle of mother spawn is sufficient .Bags which gets inoculated are shake so that the inoculums are mixed well with each other grains in order to reduce the time period for spawn preparation and to increase the quality of inoculums . For the spread of mycelium keep the bags in incubation room or in BOD incubator at 25 ^{0}C .Examined the bags during incubation for mould infestation , bags that get contaminated should be removed immediately and before discarding autoclaved the bags to avoid build- up of contamination in the vicinity . Normally it will take 20-25 days to complete spread of mycelium on grains for *Agaricus* species.

Figure 10-Inoculation of commercial spawn with master spawn

Figure 11-Pure culture , master spawn and commercial spawn in incubation room 25^{0}C

Figure 12-Ready to use commercial spawn in polypropylene bags

3.16 Spawn storage and transport

For the preparation of spawn, freshly prepared spawn should be used while the mycelium is in the state of active growth. After the completion of log growth phase the spawn bags can be maintained to 3-4 months at 4^0C. The spawn should be properly and systematically packed in ventilated bags or cardboard boxes .The spawn should be carried properly in refrigerated vans to large distances. Temperature of planter spawn does not raised beyond 30-32^0C when the spawn can be transported to long distance during night by public transport or any private vehicles. The spawn of *Volvariella* and *Colocybe* mushroom should not be stored at refrigerated temperatures because these mushrooms are very sensitive to low temperatures. At cooler temperatures the old spawn bags of *Auricularia* may also ooze brownish liquid that may result in losses of crop.

3.17 Recent advances in spawn production technology

Various steps are involved in the improvements of spawn production technology. Preparation of commercial spawn was done in milk or glucose bottles, which create problem in transportation.

Heat resistant polypropylene bags have revolutionized in the spawn industry For aeration high tech spawn labs now use polypropylene microfilm windows .However ,in Europe and USA for the production of spawn polypropylene translucent bottles of 5-10 litre capacity are also, but now it is not been introduced in India due to its high cost material. The cultivation of Shiitake mushroom was done on synthetic sawdust substrate and this technique is still practiced in developing countries. After the 120 days of incubation with solid spawn the normal fruiting bodies were harvested from colonized substrate block. The incubation time was reduced to 90 days with the use of liquid

spawn. The time incubation was reduced to 90 days by using liquid spawn. For breeding of this mushroom liquid culture can be used.

3.18 Precaution in spawn preparation

A number of precautions have been suggested as follows:

- Strict hygiene should be maintained from raising of pure culture for the storage and transport of spawn.
- Inside the inoculation room excess of visitors should be denied.
- With the help of formalin inoculation room should be disinfected regularly to eliminate chances of contamination.
- The contaminated bags should be autoclaved and buried in the soil in order to avoid contamination.
- It is important to start with a fresh spawn for each growing cycle.
- With the help of aluminum foil culture should be wrapped to prevent contamination of cultures during its storage under refrigerated conditions.
- Never store cultures and spawn at sub-zero temperatures in deep freezers. However, preservation of cultures can be done at ultra low temp using cryo-protectants proper.

3.19 Management of contamination

The most common contaminants during the spawn preparation are species of *Aspergillus*, *Penicillium ,Trichoderma, Cladosporium, Chaetomium, Alternaria , Mucor, Rhizopus, Fusarium and Drechslera*. Grains are the mains source of contamination. The fungal contamination can be easily recognizes with the typical colors of their mycelium and spores or conidia. At times a distinctive zone can be recognized as lesion between inoculated mushroom mycelium and contamination. If these contaminants are continues to grow, a large number of spwan bags may spoil. If contaminated bags are not timely removed or disinfected, it causes contamination to a great extent or level. To reduce the losses caused by fungal contaminants carbendazim or thiophanate-methyl @ 0.05g/kg of boiled grains can help. It is dough to detect the bacterial contamination. Some bacteria give a greasy appearance and emit a pungent or foul order smell. All the commercial spawn prepared from them will become useless. If the bacterial contamination is not detected in master spawn bottles, and may result into total or great loss of spawn and crop .The common bacterial contamination problem is wet spot disease caused by Bacillus species in mushroom spawn (Ahlawat et al., 1999). The

disease can be maintained by balancing the ph of spawn substrate near 6.0 and by incubating it between 20-25^{0}C.If there is no stoppage of contamination, antimicrobial compounds like neomycin, streptomycin orstreptocyclin @ 10-15 ug/gram of spawn can be added after boiling of wheat grains to control the disease.

3.20 Infrastructure and running cost

Proposed installation capacity: 2000kg/annum (20 tonnes /annum)

Today there is a lot of awareness about cultivation of mushroom in India and people are coming forward to take up commercial cultivation both as self- employment venture and also for subsidiary source of income. The good and poor quality of mushroom seeds directly affects the mushroom production. Each mushroom species has its specific seeds. Only 4 species are commercially cultivated named as *Agaricus bisporous* , *Pleurotus spp*. , *Volvariella volvacea* and *Calocybe indica* .

Table 2 -Spawn requirement for growing different mushroom species in India (2004-2005)

Mushroom species	Annual production (Tonnes)	Spawn rate per Tonne of prepared substrate / compost (kg)	Annual spawn requirement (Tonnes)
Agaricus bisporous	50,000	5	1700
Pleurotus spp	8,000	25	1000
Calocybe indica	3,000	50	750
Volvariella volvacea	1,000	25	125
Total	62,000		3575

The total annual production of mushroom in India by the end of 2006 has been estimated about 90,000 tonnes and for meeting out this demand about 5200 tonnes mushroom spawn will be required . In India, production of spawn and supplied by Research Institutes and Agricultures Universities and private spawn laboratories. Most of the units export oriented have their own spawn production facilities. The cost of the spawn is the major economic factor for cultivation of mushrooms like *Pleurotus* and *Calocybe indica*. Profit will be more if the spawn is prepared by the producer himself and one assured of the quality mushrooms.

3.21 Selection of site and layout of a spawn production unit

It is always desirable that the spawn should be isolated from composting yard and growing rooms. A separate block for spawn unit can be constructed above the office rooms.

3.21.1 Store room

Storage room is required for storage of following:

1. Grains
2. Chemicals
3. PP bags
4. PP necks
5. Cotton bundles etc.

For maximum space utilization there should be minimum 4-5 selves. The room should be 4×4×4 m size.

3.21.2 Boiling and filling rooms

The size of room should be 4×4×4 size that can be used for washing, boiling and filling water supply and drainage purpose. Near the water drainage boiling cattle or vessel should be placed

There should be one or two exhaust fans so that the grains could be dried quickly. A platform made of marble or cement should be on the opposite with grains, filling and plugging bags.

The platform should be 1.10m (h) ×0.9m (b) ×4m (l).

3.21.3 Autoclaving room

The bags after filling should brought for sterilization in autoclave room. Autoclave of 750mm×550mm size should be used for sterilization purpose. The after autoclaving bags should be kept for around 6 hours so that water droplets on the PP bags are absorbed before inoculation. These should be a window between autoclaving room and inoculation chamber. Sterilized bags are to be transported only through this window directly to the inoculation room. The size of autoclaving room should be 3×3×4 m.

3.21.4 Inoculation room

Before inoculation the sterilized bags are to be kept in the laminar air flow in a double door room which are located between autoclaving and incubation room. It should be kept for minimum 15 to 30 minutes under the UV light before inoculation. For the storage of master spawn and cultures, small BOD (6 cft) and a refrigerator is also required. The size of inoculation room should be 4×3×3×4 m in size.

Figure 13-Inoculation room

3.21.5 Incubation room

This room should be environmentally controlled for incubation of inoculated bags at suitable temperature. Iron racks with 5 tiers at a distance of 30 cm between 2 tiers, 37.5 cm wide and 1.5 cm long could accommodate 72 bags of half kg spawn in each tier. On a single rack about 350 to 360 bags could be kept. An incubation room of size 4×3×4m size could accommodate 15 racks of the above size. The number of incubation room can be increased as per the requirements of the spawn laboratory.

3.21.6 Cold room

The cold room of 3×3×4m is used for storing fully colonization of spawn bags. Cold room should be insulated and the temperature should be between 4 to 6^{o}C. The cold room have atleast1 door for taking prepared spawn out of the cold room. One air curtain should be fixed above the door.

3.21.7 Corridors

A glazed Corridor should be there of 6×2×4m at the main entry of the spawn unit .At main entry air curtain should be provided.

3.21.8 Equipments

The following major equipment's are required for commercial production of spawn:

1. Storage bins for grains (5 quintal capacity 6 Nos.)
2. Boiling and soaking pans – 4 Nos. (50 litres capacity).
3. Autoclaving (750 mm depth and 550 mm dia.)-1
4. Laminar air flow – 6'size –1
5. BOD incubator (90×90×90cm)-1
6. Refrigerator (210 litres capacity)-1
7. Racks for keeping bags (6'h×5'1×15'×b) with 5 tiers = 15 No.
8. Trolleys for transporting bags – 1 No.
9. Exhaust fans – 2(atleast)
10. Air curtain – 3 Nos.

3.22 Minor instruments

1. Spirit lamps, (2Nos.)
2. Inoculation needles (4Nos.),
3. Big size sieves mounted on iron frames (4'×4;) , (6Nos)
4. Lab sitting stools (2 Nos).

3.23 Consumables

Consumables should be purchased in bulk and the total quantity required for one month should be kept in the stores. The required consumables are wheat grain

1. Rectified spirit
2. Polypropylene bags
3. PP necks
4. Apron
5. Sleepers
6. Glucose bottles
7. Petri-plates
8. Culture tubes
9. Aluminium foils
10. Gloves
11. Non- absorbent cotton etc.

3.24 Mushroom value added products

Oyster mushroom is most perishable and starts deteriorating after few hours depending upon its storage conditions. Shelf life of this mushroom varies from 1-2 days at the ambient temperature due to high moisture content, delicate moisture and unique physiology. Hence, it is necessary to develop suitable post-harvest techniques for its prolonged preservation and usage. Indian fresh mushroom market is largely contribution of marginal and small growers with limited resources have to depend on local market for sale of their produce. Many times growers face problem of over saturation of market and distress sale at highly non-remunerative prices. The retention of fresh mushroom at various levels such as grower, whole seller, retailers and consumers further results in deterioration in quality of the produce and economic loss. Presently, long-term preservation of mushroom by drying, canning and pickling are in vogue (Chandrasekar et al., 2002). But, surplus mushroom production during peak harvesting periods can be checked by adapting appropriate post–harvesting technology to process surplus mushrooms in the form of novel value added products rather going only for drying and canning. These value added products not only help to reduces the post-harvest losses but also help in enhancing the additional incomes to the mushrooms growers and provide low fat , protein rich food to the consumers (Arumuganathan etal., 2005). Current study deals with the development of value added products of oyster mushroom like as biscuits, mushroom soup, mushroom pickle, mushroom soup, mushroom pickle, mushroom patties and pakoda.

3.24.1 Mushroom biscuits

Mushroom powder were used to make delicious and crunchy biscuits by using various ingredients such as maida, sugar, butter, mushroom powder, crushed cashew and different dry fruits, baking soda and milk powder. Preparation of dough was done by mixing all the ingredients for 10-15 minutes. After that the dough was kept as it is for 10 minutes. Therefore, thin sheets of dough was made and cut it into different shapes for making biscuits. These raw cut biscuits were kept in the trays and then these trays were shifted to micro wave oven (60^{o}C) for baking purpose for 10-15 minutes. After baking, trays were removed from micro oven and cool down. The biscuits were ready to eat, packaging and for serving.

The ingredient like sugar gives desired sweetness; butter gives smoothness to the biscuits. The various ingredients required for preparation of mushroom biscuits are as follows:

Ingredients	Quantity
Maida	100gm
Mushroom powder	150gm
Butter	250gm
Crushed Sugar	To taste
Milk	250ml
Cashew nut (crushed)	200gm
Baking Powder	5gm
Water	As required

3.24.2 Mushroom soup

Many mushroom dishes were prepared as one of the method of short term processing. For the preparation of mushroom soup firstly freshly harvested mushroom were taken and chopped into small pieces and then it get dipped into the boil water for 10-15 min for the dispersal of their spores and removal of dirty particles .Then the mushroom were washed and fried with different ingredients like onion, ginger, salt, garlic, cloves for around 10-15 minutes. The fried paste mixture was boiled for 10-15 minutes. Finally black pepper are added to the soup for garnishing and served with soup – sticks. The following ingredients were used for its preparation:

Ingredients	Quantity
Fresh Mushroom	500 gms
Onion	100gms
Ginger	50 gms
Garlic	10-15 cloves
Salt	To taste
Black peeper	To taste
Butter	200 gms
Water	1.5 litre

3.24.3 Mushroom Curry

For making mushroom curry ingredients like tomato, pea and capsicum were washed and chopped. Chopped onion, ginger, garlic was fried with the cumin seeds until it turns to golden in color and then the mushrooms, tomato and capsicum was added and again get fried well . All spices and salt added and again boiled for five minutes.

Ingredients	Quantity
Mushroom	500gm
Tomato	200gm
Onion	100gm
Capsicum	2pcs
Garlic	30gm
Ginger	50gm
Oil	As required
Salt	To taste
Turmeric	½ teaspoon
Black peeper	To taste
Chili	20gm

3.24.4 Mushroom patties and mushroom pakoda

For the preparation of mushroom pakoda freshly harvested mushroom were taken. The fruit bodies of mushroom was washed with warm and chopped into small pieces. Ingredients like onion, chilli, capsicum were also chopped into small pieces. Paste of gram flour was prepared and to it onion, chillies, salt, turmeric powder was added and mixed it properly. chopped mushroom was then dipped in the paste of gram flour and fried in hot edible oil until it get golden in color around 5-8 minutes .

Following ratio of ingredients were used to prepare to make mushroom pakoda.

Ingredients	Quantity
Mushroom	1 kg
Onion	300gm
Capsicum	2pcs
Chili	50gm
Salt	To taste
Turmeric	½ teaspoon
Gram flour	350gm
Oil	As required

The various value added product made from the fresh dried oyster mushrooms viz., mushroom biscuits , mushroom soup , mushroom patties and pakoda were evaluated for colour, appearance, flavor, taste, texture and overall acceptability by using organoleptic evaluation on the 10 point Hedonic Scale through panel of ten judges (Rangana, 1994). Data (table 3) shows that mushrooms biscuits and mushroom pickle rated the highest sensory score among the all value added product by the taste pan.

Figure 14-Innovative edible products using mushroom

Table 3- Oragnoleptic evaluation of oyster mushroom value added products

Name	Colour	Appearance	Flavour	Taste	Texture	Overall Accepatability	Mean	Storage period
Mushroom Biscuits	8.2	7.7	8.2	8.6	8.4	8.7	8.3	12 months
Mushroom soup	6.9	6.2	7.7	7.6	6.9	7.2	7.2	--
Mushroom Jam	-	7.8	8.0	8.4	7.9	8.2	8.2	8.06 months
Mushroom Pickle	8.6	8.3	8.2	8.1	8.1	8.3	8.3	12 months
Mushroom patties	7.9	7.7	7.5	8.3	8.3	7.9	7.9	--
Mushroom pakoda	8.4	8.2	8.5	8.6	7.5	8.2	8.2	--

As far as quality is concerned that the oyster mushroom, pickle was very good in colour and texture, quality, appearance, flavor, taste, flavor. Shelf life of both mushroom pickle and mushroom biscuits , storage quality concern in general and colour, taste , texture and appearance in particular does not affected for a period of 12 months . The oyster jam, novel product was also liked by the panel of judges in both overall quality and storage concern and reported maximum storage life of 6 months. Taste and appearance of mushroom jam also did not changed during the course of study. Mushroom jam colour i.e. light brown, remained unchanged throughout the storage period. The other product mushroom patties and pakoda had excellent taste, flavor, taste and appearance and were also liked by panel of judges. Mushroom soup ranked the lowest sensory score rated from 6.2 to 7.8 with respect to colour, appearance, flavor, taste and texture. However, overall quality of mushroom soup was reported to be above the acceptability level by the panel of judges. With the regard to taste , the product named mushroom biscuits and mushroom pakoda ranked the highest and equal sensory score (8.6), mushroom pickle and mushroom jam and mushroom soup ranked third (7.9) and lowest sensory score (7.6), respectively . The texture and flavor of the products , which are a deciding factor of the products acceptability , were good in general and ranked from 6.9 to 8.4 , 7.5 to 8.5 ; respectively . As far as the overall acceptability concerned, mushroom biscuits topped in list, followed by mushroom pickle, mushroom patties and mushroom soup. Thus preparation of mushroom value added products not only adds the value and returns the additional income to the mushroom growers but also provides protein rich nutrients food to the consumers.

3.25 Spent mushroom substrate for crop

After the cultivated mushroom fruit body has exhausted the nutrients within the substrates and there is no more fruit body to grow, the substrate so called remains is known as spent mushroom substrate. Spent compost or substrate is rich in organic matter and constitute an important source of macro and micronutrients for plants and micro-organisms thereby increase the soil micro-flora, soil biological activity and enhance soil enzyme activity. This spent compost can be used as soil conditioner or organic fertilizer to improve the plant health status. There are many ways to use the spent mushroom substrate. SMS is excellent to spread on top of newly seeded lawns.
The SMS provides cover against birds eating the seeds and will hold the water in the soil while the seed germinate. Since some plant and vegetables are sensitive to high salt content in soil, avoid using fresh substrate around these plants. In these case 6 months or more weathered spent substrate can be used. As a soil amendment, SMS adds organic matter and structure to the soil. Total four field trials have been conducted to evaluate the effect of spent mushroom substrate for improvement of plant health status.

3.26 Determination of colony forming unit (CFU) in dry waste of mushroom

Prepare nutrient agar media (bacterial count), by taking the composition of 28 gms of nutrient agar in 1000 ml of distilled water in a conical flask , actinomycetes agar (actinomycetes count) , by taking the composition of 21.7gm in 1000ml of distilled water and Rose Bengal Choromophenicol Agar (fungal count), by taking the composition of 32.15 g in 1000 ml of distilled water then put all the media it in a autoclave at 121^0C of temperature and 15 lbs of pressure for 15 minutes maintaining the pressure. After removing content from the autoclave let it to cool a slighter then start with the pouring of media onto the petriplates by flaming the mouth of it and then let the media to settle into it and solidify, then closing the mouth of the petriplates let it to be in the laminar hood. Prepare the samples for serial dilution by taking 1gm of sample and taking 9ml of distilled water in the tubes. Label the tubes with the dilution factor order. Transfer the sample into the tube of dilution factor 10^{-9} and taking 1ml from the 1^{st} tube and transferring it to other using a micropipette and then taking its 1ml to transfer into the other continuing this process preparing tubes of dilution factor 10^{-9},10^{-8},10^{-7},10^{-6},10^{-5},10^{-4},$10^{-3.}$ With the help of pipette 100µl capacity of sample putting on the petriplate on it spreading the inoculums on the nutrient agar media, actinomycetes agar media and in Rose Bengal Chromophenicol Agar media , using a glass spreader and moving the petriplate in circular motion clock wise and then anti clockwise. Now cover the petriplates and let the inoculum to be absorbed in the media after that inward the petriplate and put it in the incubator for 24 hrs. of incubation period at temperature of

28 ^{0}C .After the completion of incubation period observe the bacterial and fungal growth in the medium by recording the no. of colonies onto the media in petriplates.

Figure 15-Placed the petriplates in incubatator for 24 hours at 28 ^{0}C for bacterial and fungal growth.

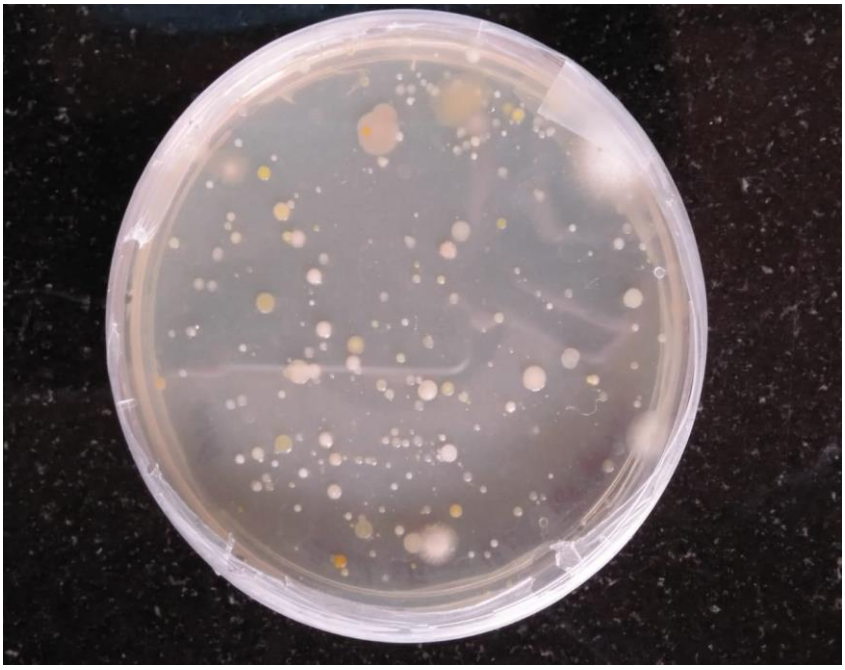

Figure 16-Colonies of actinomycetes on actinomycete agar media plate

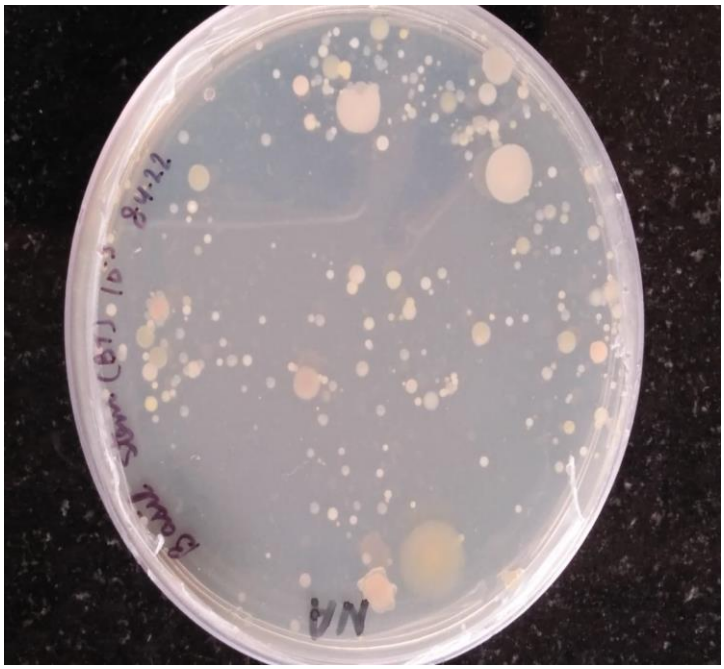

Figure 17-Colonies of bacteria on nutrient agar media plate

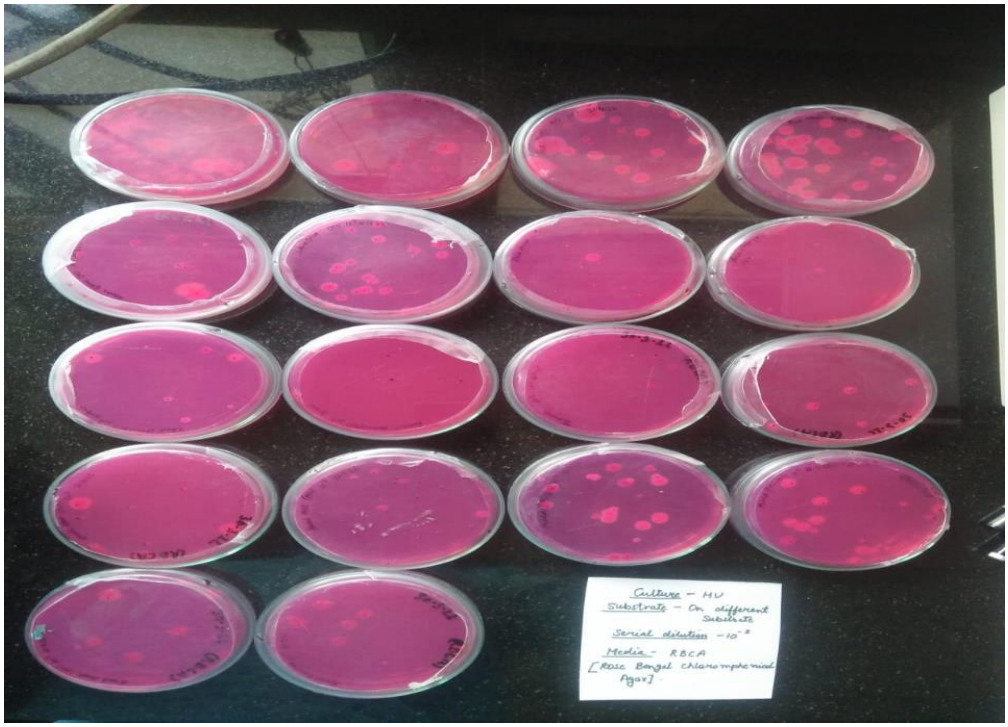

Figure 18-Colonies of yeast and mould on Rose Bangal Choromaphenicol agar plate

Result

4.1 Study on mycelial growth pattern of *Pleurotus spp*

Media such as potato dextrose agar were used to evaluate the mycelial growth rate and growth pattern of four species of oyster mushroom (*Pleurotus)*.Results have been presented in below table 11 and figure 18. The four cultures HU, BT, PF and PO were used and incubated at 24-25^{O}C. It was observed that among all four culture BT grew faster than HU, PF,PO. Initially the mycelial mats grew very thin and later the mats became cottony. *Pleurotus* showed white cottony growth of mycelial mat in concentric manner.

Table 4- Study on growth rate and growth pattern of oyster mushroom on PDA media

Oyster Mushroom (Culture)	**Media**	**Growth initiation (hours)**	**Mycelial colour**	**Texture**	**Mycelia growth (cm)**			
					48 hrs.	72 hrs.	96 hrs.	120 hrs.
BT	PDA	24 hrs.	White	Cottony	0.9	2.3	3.7	5.4
HU	PDA	24 hrs.	White	Cottony	0.7	1.7	2.9	4.3
PO	PDA	24 hrs.	White	Cottony	0.7	1.9	2.8	4.9
PF	PDA	24 hrs.	White	Cottony	0.5	1.9	2.9	4.1

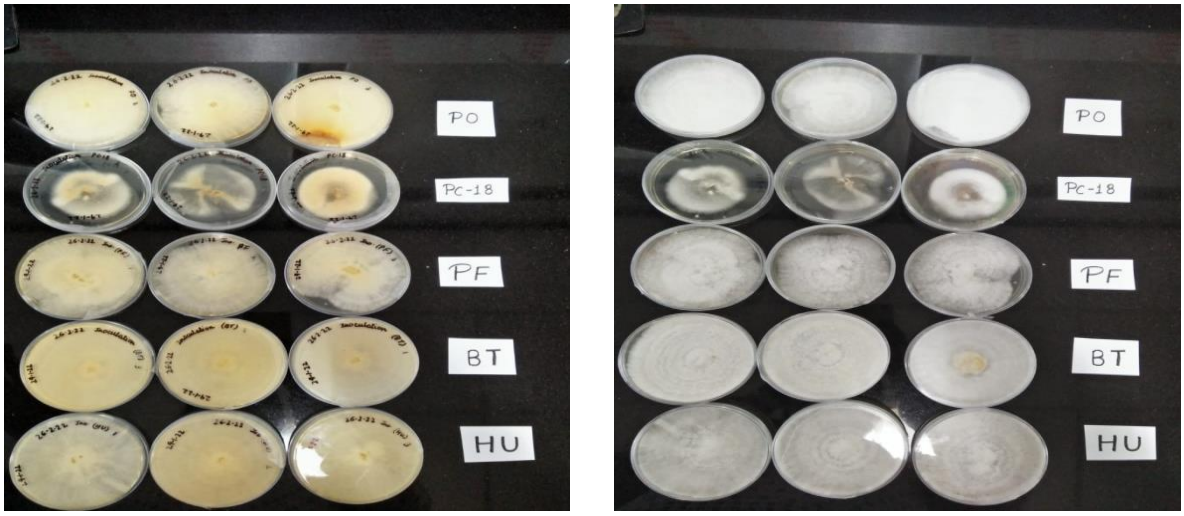

Figure 19-Growth pattern of culture PO, PF, BT, HU after 120 hours

4.2 Morphology and Histological study of sporocarp of edible oyster mushrooms (*Pleurotus spp.)* -

Pileus of oyster mushroom was thick, fleshy, very short stipe. Diameter of the young pileus was about 7-10 cm and the whole part of the fruiting body was edible.
Anatomical study of *Pleurotus spp*. revealed that the basidiospores were long, oval shaped quite prominent edges. Basidiospores were attached with the basidium by the corner of the basal part of the spores *Pleurotus djamor* was a new introduction of oyster mushroom in North Bengal. It looks very gracious on bed; pileus is pink in colour with very small stipe. Sometimes stipes was absent. The diameter of pileus was about 10-15 cm, fleshy with light aroma.

Figure20- S2 (BT)

Figure 21-S1 (PO)

Figure 22-S3 (PF)

Figure 23-S6 (BT)

Figure 24- Oyster Mushroom before harvesting

4.3 Cultivation of oyster mushroom

4.3.1 Yield performance of four species of *Pleurotus* (oyster mushrooms) on different substrate -

Yielding performance was done with 4 different culture on 9 different substrate .The culture used are PO, PF, BT, HU on different substrate they were S1 to S9.

Mycelium colonization of *Pleurotus spp.* on different substrate was different. In case of strain BT days of colonization occur in S4 was in 12 days, in S9 it occurred in 13 days and in S1 it proliferated in 20 days while in S8 it took 21 days, the maximum days to spread colony of fungus. Days of pinhead appearance in S1 was in 43 days, in S4 27 days and in S9 34 days and S5 took 48, which were the maximum days to appearance pinheads taken by any other substrate. Length of mycelium in S1 was 6cm, in S4 it was 6.5 which was the maximum length among all the substrates and in S9 it was 6 cm and the S2 was of 2 cm in length, which was the smallest size stalk of mycelium among all the substrate. The diameter of caps in S1 was 11 cm, in S4 was 12 cm in case of S9 it was 8 cm while in case of S8 it was around 6cm in size which was smallest among all of them. Average weight / kg of substrate S9 was 1.929 kg which was the highest weight of oyster harvested among all the substrate, in S4 it was 0.353 kg, S1 it was 0.765 kg, which was the second highest harvested oyster among all the substrate and least harvested oyster was seen in S5 (0.048). The protein content in S6 was 2.1435%

(g/100g of dry substrate) which was the highest protein content among all the substrate and the lowest was found in S7 1.6804% (g/100g of dry substrate).

In case of strain HU, colonization was occur in S4 in 12 days , in S9 it happened in 13 days and in S1it proliferated in 20 days as compared to S8 which took 21 days, which was the maximum days to spread colony of fungus. Days of pinheads appearance in S1 was in 30 days, in S4 31 days and S9 took about 31 days for the appearance of pinheads. However, S7 spread was happened in 41 days, which is the highest days for the appearance of pinheads and second highest was S3, which took around 35 days. The length of mycelium in S4 was 3 cm, in S9 it was 4.5 cm and in S1 it is 5 cm which was the highest length among all the substrate and the lowest length was found in S5 2 cm. Maximum cap diameter was found in S1was 8.5 cm followed by S9(8cm) as compared to other substrate . The highest weight / biomass of oyster mushroom was recorded in S1 (0.904g) followed by S9 which was 0.678g. The lowest yield was recorded in S2 0.105g. Highest protein content was found in S8 which was 2.0877 and the lowest was found in S4 that was 1.3560 % (g/100g of dry substrate).

In case of strain PF, colonization was occur in S4 in 12 days , in S9 it happened in 13 days and in S1 it proliferate in 20 days as compared to S8 which took 21 days, which was the maximum days to spread colony of fungus. Days of pinheads appearance in S1 was in 41 days, in S4 31 days and S9 took about 41 days for the appearance of pinheads. However, S1, S7, S9 spread was happened in 41 days, which is the highest days for the appearance of pinheads and second highest was S3, which took around 37days. The length of mycelium in S4, S9, S7 was 3.5 cm which was the highest length among all the substrate and the lowest length was found in S1and S7 was 2 cm. Maximum cap diameter was found in S6 and S1 was 6 cm and lowest was found in S9 and S8 4 cm. The highest weight / biomass of oyster mushroom was recorded in S5 (0.996g). The lowest yield was recorded in S7 was 0.087g .Highest protein content was found in S9 which was 2.1878 and the lowest was found in S2 that was 1.4592 % (g/100g of dry substrate).

In case of strain PO, colonization was occur in S4 in 12 days , in S9 it happened in 13 days and in S1 it proliferate in 20 days as compared to S8 which took 21 days, which was the maximum days to spread colony of fungus. Days of pinheads appearance in S1 was in 37 days, in S4 29 days and S9 and S8 took about 46 days for the appearance of pinheads which was the second highest days for the appearance of pinheads and highest was S3 which took around 51 days. The length of mycelium in S4 and S8 was 2.5 cm, in S9 it was 4cm and in S1 it was 3.5cm, but in S2 and S6 was 4.5cm which was the highest length among all the substrate. Maximum cap diameter was found in S3, S9 was 6.5cm. The highest weight / biomass of oyster mushroom recorded in S1

0.827 and the lowest was recorded in S7 (0.142). Highest protein content was found in S7 which was 2.0127 and the lowest was found in S1 that was 1.3452 % (g/100g of dry substrate).

Figure25-Harvested Oyster Mushroom

Effect of different substrates on growth , development Pin head formation, and yield of Oyster mushroom on culture BT , PO , PF , HU

A) Table 5–Culture- BT

Substrate	Days of colonization	Days for pinheads appearance	Stalk Length of mycelium (cm)	Diameter of cap (cm)	Average weight / kg of substrate	Protein content % (g/100 g of dry mushroom)
S1	20	43	6	11	0.765	2.0354
S2	19	42	2	7	0.392	1.8500
S3	19	42	3	7	0.184	1.9772
S4	12	27	6.5	12	0.353	1.8760
S5	19	48	5	7	0.048	1.9623
S6	14	33	6	7	1.264	2.1435
S7	11	31	5.5	7	0.112	1.6804
S8	21	51	5	6	0.174	1.7108
S9	13	34	6	8	1.929	1.7865

B) Table 6-Culture –HU

Substrate	Days of colonization	Days for pinheads appearance	Stalk Length of mycelium (cm)	Diameter of cap (cm)	Average weight /kg of substrate	Protein content % (g/100 g of dry mushroom)
S1	20	30	5	8.5	0.904	1.9782
S2	19	36	3	8	0.105	1.7518
S3	19	35	4	6	0.259	1.4562
S4	12	31	3	6	0.305	1.3560
S5	19	30	2	5	0.226	1.9925
S6	14	29	3	4.5	0.192	1.5673
S7	11	41	3	5	0.152	1.8975
S8	21	30	3	4	0.168	2.0877
S9	13	31	4.5	8	0.678	1.7172

C) Table 7-Culture -PF

Substrate	Days of colonization	Days for pinheads appearance	Stalk Length of mycelium (cm)	Diameter of cap (cm)	Average weight / kg of substrate	Protein content % (g/100 g of dry mushroom)
S1	20	41	2	6	0.696	1.9726
S2	19	34	3	5.5	0.158	1.4592
S3	19	37	3	5	0.156	2.0155
S4	12	31	3.5	4.5	0.498	2.0388
S5	19	41	3	4.5	0.996	1.6632
S6	14	40	2.5	6	0.147	1.5673
S7	11	31	2	5	0.087	2.0188
S8	21	41	3.5	4	0.189	1.6826
S9	13	41	3.5	4	0.392	2.1878

D) Table 8-Culture-PO

Substrate	Days of colonization	Days for pinheads appearance	Stalk Length of mycelium (cm)	Diameter of cap (cm)	Average weight /kg of substrate	Protein content % (g/100 g of dry mushroom)
S1	20	37	3.5	5.5	0.827	1.3452
S2	19	37	4.5	5	0.483	1.6695
S3	19	51	3	6.5	0.0765	1.9300
S4	12	29	2.5	4.5	0.3935	1.9502
S5	19	-	-	-	0	-
S6	14	31	4.5	6	0.706	1.1002
S7	11	29	4	5	0.142	2.0127
S8	21	46	2.5	5	0.379	1.8427
S9	13	46	4	6.5	0.421	1.6957

S1-S9 – various agricultural waste

4.4 CFU (Colony Forming Unit)

FORMULA USED –Number of colonies on the plates / Amount spread × dilution factor

[Amount spread - 100µl (0.1)]

[Dilution factor – 10^{-3}]

Table 9– Mean number of colonies (CFU) using different media

Substrate	Total bacterial colony on NA (10^4)				Total actinomycetes colony on AG (10^4)				Total fungal colony on RBCA (10^4)			
	HU	P0	PF	BT	HU	PO	PF	BT	HU	PO	PF	BT
S1	108	100	250	508	706	126	708	600	8	10	11	7
S2	188	162	440	278	666	138	408	348	17	12	10	13
S3	756	199	358	72	50	386	110	50	11	8	34	4
S4	726	212	326	350	94	278	112	62	20	8	11	6
S5	536	247	358	600	52	448	176	178	5	20	9	8
S6	154	272	200	589	306	272	368	232	12	8	20	19
S7	550	549	106	1128	294	610	226	214	10	16	21	28
S8	500	184	348	404	336	184	338	260	24	6	14	23
S9	796	1002	1968	450	402	1002	536	282	5	10	18	10

NA – Nutrient Agar

AA – Actinomycetes Isoloation Agar

RBCA – Rose Bengal Choromophenicol Agar

4.5 Disease occur during cultivation of Pleurotus spp

1. **Bacterial disease**
2. **Fungal disease**
3. **Viral disease**

During mushroom cultivation bacterial and fungal disease are the common and major problem. High percentage of products get lost due to lower productivity, quality decrease and shelf life get shortened.

4.6 Bacterial diseases

During the first or second sporophore flush, bacteriosis a disease that usually occur and it can cause great loss in yielding.
The destructive disease levels are held by environmental conditions at high relative humidity in growing chambers. Conditions like excess of water in the casing layer and a low aeration rate in the growing-house are Favorable to bacterial blotch outbreaks.
Yellow lesions on *P.ostreatus* sporocarps are associated with *Pseudomonas spp*. and *P.reactans* which are pathogenicity assays and have caused yellow discoloration of the sporocarps.
Environmental control which includes low relative humidity, temperature, level of carbon dioxide, cleaning of cultivation rooms all these play important roles in diminishing the spreading of the many disease in mushroom.

4.6.1 Bacterial yellowing

The diseases get observed during the cultivation of *Pleurotus eryngii* .Yellowing can cause severe damage in any growing procedure.
The appearance disease is characterized by a yellow discoloration of the pileus and hydropic, often elongated and coalescing areas on the entire portion of the stem. Disease in sporophores gives alcohol like smell and pleasant at first but rapidly it becames nauseating. Bessette et al1985 reported that yellow blotch caused by *P.agarici* in *P.ostreatus* and at first it formed a clean yellow fluid on the surface of the cluster and then it deformed with an increase in severity. The most commonly utilized chemicals for control of blotch disease are lowering relative air humidity , watering with low concentration of chlorine solution (calcium chloride and chlorinated compounds .To control the bacterial blotch disease many other disinfectants and antibiotics are used such as chloramines T and bronopol, essential oils, kasugamycin.

Figure 26-Bacterial yellowing disease in oyster mushroom

4.6.2 Bacterial brown blotch

Bacterium *P.tolaasii* is responsible for causing bacterial blotches in the button mushroom, *Agaricus spp.,* in *Flamulina spp.,* in oyster mushroom *Pleurotus spp.,* and in Shitake *Lentinus edodes*. It is one of the most serious bacterial diseases for the oyster mushroom. The disease occurs over a large geographical area .If the disease occur on a farm area, it became tough to control so it has been suggested to remove all of the substrate from the farm.

Tolassin produced by *P.tolaasii* has proven the major virulence factor. When *P.ostreatus* develops the brown blotch symptom, it continues to rot due to a volatile toxin, tovsin, produced by *P.tolaasii* only when in contact with *P.ostreatus.* The pathogen causes blotch symptoms on the pileus, forming membrane pores and disrupting the cellular membrane structure. The indication of disease is done by the formation of brown lesions on mushroom caps bacterial growth in and discoloration on the stipes. These lesions are slightly concave spots, which can be round or spreading. At or near the edge of mushroom caps spotting occur.

Mushrooms after watering remains wet even after the 4-6 hours after harvesting, blotches occur. The brown spots and blotches get enlarge and coalesce with others. The area which gets affected get sucken and covered with sticky material .This disease affects only the top outer layers of the pileus tissues and is restricted to 2-3 mm below the surface of pileus.

Bacteria may reach and colonize the outer surface of the pileus during development stage of early fruiting bodies while the young pileus was still in the contact with the substrate. If the bacterial populations are greater from spawned substrates it causes more severe infection. It is very essential to control or stop the transfer of pathogen from the spawned substrate to the pileus during the development stages of early fruiting bodies in order to control the disease.

Rapid spread of the bacterial pathogen can cause huge economic damage in *P.eryngii* cultivations and effective biological or chemical control measures are scarce. Some

Pseudomonas isolates were screened for their antagonistic ability toward *P. tolaasii*; and there in vivo suppressive effect was not satisfactorily proven.

Figure 27-Bacterial brown blotch disease in oyster mushroom

4.6.3 Soft rot

The genus *Pantoea* includes several species that are associated with plants, either as epiphytes or as pathogens. The gram-negative bacterium *Pantoea spp*. has been reported as a causal agent of soft rot disease with symptoms of water-soaked lesions on the stipes and pileus of *P. eryngii.* The symptoms of soft rot disease include the early stages of infection, dark brown water drop followed by the development of water-soaked lesions on the stipe and cap of mushrooms within the 8 days after mushroom get transferred to cultivation room. The expansion of lesion occur gradually and constitute mucus like fluid which leads to mushy soft rot accompanied by odor during growth. The strains belongs to *Pantoea beijingensis*, growth occurs at 10-37^{0}C from lesions on the fruiting body of *P. eryngii*, exhibiting the symptoms which include water-soaked lesions, soft rot in stipes also in pileus. Most common chemical utilized by bacterial disease control are compound containing active chlorine. Watering at the concentrations at 175 ppm active chlorine were effective for the reduction of soft rot disease of *P. eryngii* without damaging the fruit bodies of mushroom .

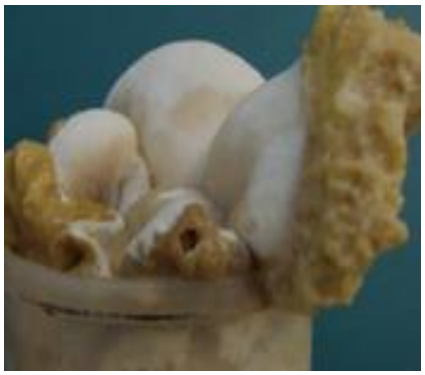

Figure 28-Soft rot disease in oyster mushroom

4.6.4 Stipe necrosis

Ewingella americana was identified as a pathogen of Stipe necrosis Reyes et al.2004 demonstrated that the predominance of *E. americana* in biota of retail fresh *P.ostreatus*. In the centre of mushroom stipe the symptoms of internal stipe necrosis appear as a variable browning reaction. During the time of examined in longitudinal section, the brown tissue extends from the base of the stalk to the cap, but rarely it get penetrates to the tissue of cap .The mushroom which get affected appears as a wet but frequently, at harvest the brown tissue is dry and get completely collapsed as a result hollow is appeared at the centre.

Mostly, the symptoms are visible only at the time of harvest. The internal stipe necrosis disease has associated with water-logging of the mushroom stalks at as early development stage .The occurrence of internal stipe necrosis disease has occasionally been associated with water-logging of the mushroom stalks at an early development stage. It is very important to maintain proper evaporation from the bed surface at all times.

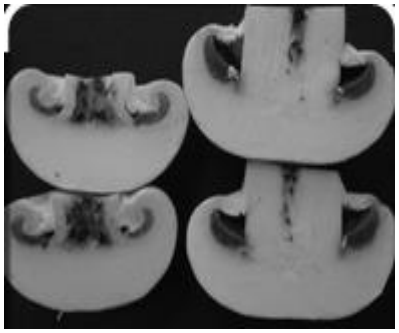

Figure 29-Stipe necrosis disease on mushroom

4.7 Fungal diseases

4.7.1 Dry bubble

Lecanicillium fungicola is a pathogen which causes significantly losses in the commercial production of *Pleurotus spp*. in the mushroom industry. In commercially cultivated mushroom this mold causes dry bubble disease. The *L. fungicola* is not found on wild mushroom, have no wide host range and might more often infect already decaying mushrooms. At the time of infection two distinct symptoms are been observed on the development stages of the sporophores. Infection of sporophores button stage resulted in the development of typical dry bubbles and masses of sporophore tissue. The mature sporophores show cracking and curling of the tissues. At the advanced stages, a gray weft of mycelium and conidia covered the surface of infected sporophores. Strict hygiene, environment regulation, daily fungicide spray program can control the *L,fungicola* . Many chemicals like Sporogon are used to control dry bubble because hot is also sensitive to fungicide. *L.fungicola* to Sporogon sensitivity has decreased therefore concentration of Sporogon must be increase to combat dry bubble disease.

4.7.2 Green mold

The production of oyster mushroom commerically affects the green mold epidemics. The causal agents of such disease of cultivated oyster mushroom such as *Trichoderma spp*. *Trichoderma* species are usually asexual and soil-inhabiting filamentous fungi belonging to the genus Hypocrea. *Trichoderma pleurotum* has been found only on cultivated *P.ostreatus*. The *Trichoderma pleuroticola* are found both on wild and cultivated *P. ostreatus* (oyster mushroom), as well as on the natural and productive substratum. In edible basidiomycetes, *Trichoderma* green mold infection has been known for a long time. In oyster mushroom substrates the appearance of green fungal sporolation are green mold symptoms.

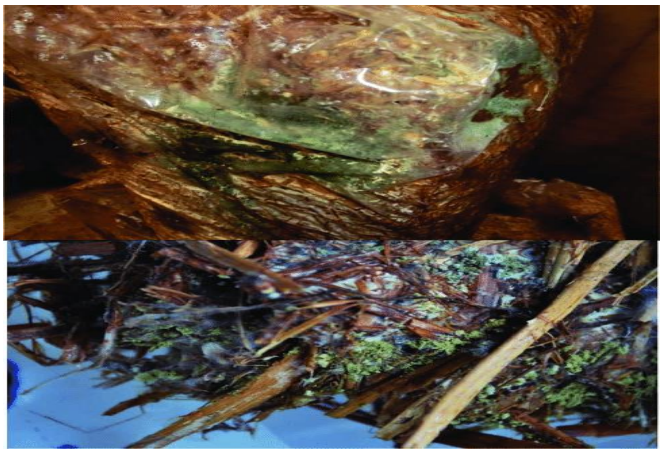

Figure 30-Green mold disease during fruiting-bodies of oyster mushroom

4.7.3 Cobweb

The causal agent of Cobweb including *C. dendroides*, *C. mycophilum*, *C. varium*, *C. multiseptatum*, and *C. verticillatum*. However, in 2009-10, some commercially grown *P. eryngii* began to show similar symptoms to the fungal disease caused by Cladobotryum mycophilum in *A. bisporus*. The spores are large and multicellular in nature, being easily dislodged from the sporulating colony by external disturbances such as watering, air circulation systems, and harvesting. Conidia were able to germinate and grow within 4 hours. Spores are very quickly dispersed in the air and growth of conidia is an important causative factor of cobweb disease in *P.eryngii* suggested by facts .The spores which are air borne affects the crops which may contaminate reusable plastic bottles, substrates and it can even spread quickly from farm to farm. This is main way of transmission of pathogen. Cobweb-like growth of fungal mycelium over the mushroom is one of the main symptoms of this disease. The colonies rapidly spread over the mushroom and it can develop spores within the few days (3-4 days). The mycelium covers the king oyster mushroom debris, pin-heads, stalks, caps, pileus and gills quickly. Result in decomposition of the complete fruit body. The colonized surface turns into pale brown or yellow. The fruit body turns dark brown and becomes rancid, with an offensive odor smell.

Figure 31-Cobweb disease on button mushroom

4.8 Viral diseases

In viral disease the symptoms were observed in the culture when a certain mushroom spawn was inoculated. Brown blotch disease which is a bacterial disease by *P.tolaasii* was restricted to a local area of culture. This leads to a result that the symptoms originate from virus-infected spawn. Retarded mycelia and fruiting body growth, fruiting body growth, inhibition the development of fruiting bodies and malformations of the fruiting bodies are the symptoms of mycoviral infections.
In 1980 *P.pulmonarius* virus was mycelia isolated and basidiocarps of *P. pulmonarius. P.eryngii* showed symptoms of fruiting bodies such as short and stout stems and caps are flatterened with irregular shapes. If the viruses are cured they eliminate the symptoms of causative agents of the disease. Therefore, it is conceivable that many of the unknown disease of mushroom may be related to mycoviral infections.

4.8.1'La France'' disease

The oyster mushroom isometric virus OMIV was isolated by Ro et al.2006 .When the characteristics of OMIV was get examined it was suggest that it is not related to known *P.ostreatus*-infecting viruses like PoV1, PoV1 is a spherical in shape that consist of two dsRNA genomes and have diameter of 30nm.The symptoms of viral disease on oyster mushroom are almost similar to that of La France disease. In *Agaricus bisporous* formation of fruiting bodies delay, stipe shorten, thin caps of mushroom and abnormal shape are the major symptoms. It is found that fruiting bodies are not formed on some infected mushroom and on agar media the viral-infected hyphae grow slowly and their density is low.

Figure 32-'La France' disease on mutton mushroom and oyster mushroom

4.8.2 Die-back

The cultivation of *Pleurotus spp.* is done under well-controlled environmental conditions and its cultivation is largely free from disease from external origin .Industries of mushroom often suffer from spawn-related diseases , mostly the die-back disease which usually originates or spread from viral infection . Yu et al.2003 isolated the first single-stranded ssRNA mycovirus , named (OMSV) oyster mushroom spherical virus , from a cultivated oyster mushroom .The author detected viruses from 102 different commercial farms with the epidemic in all 102 samples . The symptoms are usually complex and the spreading of disease is faster. The outbreaks of these disease leads to complete loss of yielding which is difficult to control in a commercial farm . However, the OMSV was not easily detected in healthy mushroom and when OSMV from diseased mushroom get cured the epidemic generally disappeared. This shows that OSMV is a causative agent of the diseased. Another spherical dsRNA virus was discovered infecting the *P.ostreatus*, being named PoV143.

Figure 33- Die back disease on mushroom

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